STUDIES ON CELL STRUCTURE BY THE FREEZING-DRYING METHOD

V. THE CHEMICAL BASIS OF THE ORGANIZATION OF THE CELL

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ONE FIGURE

In 1835, Dujardin wrote concerning his investigations of the properties of living matter the following description of his sarcode:

I propose to name sarcode that which other observers have called a living jelly. This glutinous, diaphanous, homogeneous matter, reflecting light a little more than water but much less than oil; capable of being stretched and retracting itself like mucus, elastic and contractile, susceptible of producing spontaneously spherical cavities or vacuoles occupied by the surrounding liquid. . . . Sarcode is insoluble in water. In the long run, however, it decomposes, leaving a granular residue. Potash does not dissolve it suddenly like mucus or albumin; it appears simply to hasten its decomposition by water. Nitric acid and alcohol coagulate it suddenly and and render it white and opaque. Its properties are thus very distinct from those of substances with which one might be able to confuse it, for its insolubility in water distinguishes it from albumin and its insolubility in potash distinguishes it at the same time from mucus and gelatin, etc. The most simple animals—amoeba, monads, etc.—are composed solely, at least in appearance, of this living jelly.

The key to the solution of this riddle of the difference between protoplasm and simple solutions of proteins has been in our possession for over 50 years, since it depends upon the

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presence in protoplasm of a peculiar substance which by its properties is responsible for the integrity of the cell as a unit of structure and for its internal organization. We refer to the discoveries of Reinke and Rodewald, published in 1881, concerning the chemical composition of the protoplasm of Aethalium plasmodia. By pressing out fresh protoplasm of Aethalium in a powerful press, these observers obtained a residue from which no further protein could be obtained either by means of 0.2 per cent potash or 0.2 per cent HCl. This material the authors term 'plastin,' and regard it as the basis of the framework of the protoplasm of this organism. Later investigations, particularly by E. Zacharias, O. Hammarsten, F. Schwartz, Carnoy, and A. Schmidt,[2] confirmed the thesis of the general occurence in living substance of a protein material less soluble in the usual solvents than the ordinary protein material.

These classical works which probably constitute the most important contributions ever made to the study of the living matter have been so thoroughly forgotten that modern works on cytology scarcely mention them. The reason for this probably is to be found partly in the fact that findings were referred largely by the authors to the older, now discredited, architectural theories of protoplasmic structure, partly to the fact that investigators lacking confidence in the pictures obtained by even the most modern technique have endeavored to deduce the structure of protoplasm from the general properties of colloids and from newly discovered facts of physical chemistry rather than from the facts acquired by observation. The theme has suffered from too much sophistication and too little critical repetition of fundamental experiments. It has been our unique privilege by the application of the freezing-drying method to rediscover this sovereign substance of protoplasm by methods which are less open to criticism by reason of the fact that it was not necessary to use agents which coagulated or agents which corroded.

[2] A complete bibliography of this literature will be found in E. Zacharias, "Die chemische Beschaffenheit von Protoplasma and Zellkern," Progressus Rei Botanicae, vol. 3, 1910, pp. 67–257.

In the preparations upon which the four previous sections of this series were based, it was noted that whether the cells of the Amblystoma liver were extracted with water, with salt solution, or submitted to treatment with acids, or artificial digestion with gastric juice, there was still a residue of the cell retaining its original form and for the most part its original structure.

We had doubts as to the validity of this observation, because at the time when these reports were published we did not actually know the extent to which the solubilities of constituent substances of protoplasm were modified by the freezing-drying procedure. For that information we depended upon previous biochemical investigations. We were confronted, however, by the fact that in the case of mitochondria contrary results were sometimes obtained. In some preparations the mitochondria of the liver of Amblystoma were immediately soluble in water or salt solutions, while in other preparations made in exactly the same way the mitochondria were quite refractory to these reagents. The tentative explanation was made that there was a slow irreversible gelation of the mitochondrial substance. The alternative hypothesis that mitochondria actually differed in solubility at different moments of the cell life was not considered. In the preparation upon which this description was based the material was embedded in paraffin and we were unaware what effect this embedding had upon the solubilities of intracellular constituents. For this reason it seemed necessary to determine directly the effect of the freezing-drying technique upon these properties. The only way by which this could be done was by rejecting all embedding and examining the material directly from the vacuum apparatus after drying, using the free-hand section method to obtain the necessary preparations. The liver of Amblystoma lends itself very well to this method, because it acquires a consistence in the process of drying that enables one to cut fairly respectable sections with the free hand.

Free-hand sections of this sort from material immediately after removal from the drying apparatus, floated on 0.7 per cent solution of sodium chloride, became immediately surrounded by a zone of dissolved protein substances and glycogen. If the section were so floated on the smallest drop of water or salt solution possible and immediately dried, tests of this material with Millon solution showed an area around the section which gave an intense Millon reaction, or, tested with iodine water, the same area gave an intense glycogen reaction. This indicated that both glycogen and the cell globulins had retained their solubility. Similar preparations so treated with water and salt solution and dried on the slide, fixed by momentary exposure to osmic acid and stained with anilin-acid-fuchsin and methyl green, were completely lacking in mitochondria. The nuclei, unless the nucleus had been cut in two by the section, were little affected by the solvents. Sections of the same sort, floated on osmic acid and dried and stained as before, give no loss of material by solution and perfect preservation of the mitochondria.

The material available for this investigation was then divided into two portions: One portion was placed in a refrigerator at 2°C., and the other kept on the laboratory table. In the preparation kept on the laboratory table the solubilities of the mitochondria had been completely abolished after a period of 2 weeks. The same material which showed complete solubility of the mitochondria in salt solution had lost this solubility after embedding in paraffin and sectioning. The preparations kept in the refrigerator at low temperatures maintained their solubilities longer, but at the end of 4 months in them, too, the solubility of the mitochondria was lost.

In frozen-dried material not subjected to the action of any coagulating or fixing reagents kept in the light and air at laboratory temperatures for a period of 2 weeks, the mitochondria had been so profoundly changed in their solubilities that they were not only not soluble in water and salt solution, but they were not even soluble in a tenth normal solution of ammonia in which the rest of the cell literally dissolved. In

this way smears were made in which the only recognizable formed element remaining were the mitochondria which had become so insoluble that they were not even altered in their form nor in their staining properties.

Having thus established the retention of the solubilities of certain intracellular proteins in 0.7 per cent salt solution up to the moment of removal from the cold vacuum, it was possible to wash free-hand sections of the liver of Amblystoma completely free from mitochondria and from soluble globulins. When the removal of these substances was accomplished, the cell still retained its shape, its integrity as a unit, and its internal structure as before. This fact indicated that the integrity of the cell as a unit rested upon the materials not soluble in normal salt solution.

The same result was obtained in pieces of Amblystoma liver prepared by the freezing-drying method which were immediately placed in salt solution or other solvents. The latter were allowed to act for a certain time, after which the preparations were fixed in formalin and embedded in paraffin and sectioned. These pieces gave a gradient of solution of cell constituents from the outside of the block to the interior, and if the time had been properly estimated every stage in the alteration of the cells by a solvent could be discerned. In such preparations treated with 0.7 per cent sodium chloride the cells of the outermost layer of the section showed a complete structure, but lacking both mitochondria and soluble globulins. In the intermediate zone between the outside and the inside, the globulins were released from their prison in the cytoplasm and reprecipitated as a granular precipitate while the mitochondria were rounded up to vesicles such as were illustrated in the previous description by Bensley and Gersh ('33). In the innermost layer, if the time of immersion had not been too long, the unaltered cells were apparent, a perfect gradient being established between all of these stages.

Since it was necessary to turn to a material which was more abundant than that of the liver of Amblystoma, similar experiments were carried out with guinea pig liver and with

rabbit liver, where, to our surprise, it was found that although the globulins and glycogen were extracted from the cells with equal ease, the mitochondria were wholly insoluble in either water or salt solution, or neutral Ringer's solution. This coincides with our previous experience that, while in the Amblystoma liver the mitochondria were soluble directly in salt solutions, in many other tissues this solubility was not present.

Rabbit liver freshly dried in the frozen state was ground up to a flour in a mortar, then suspended in salt solution. This material was then repeatedly extracted with fresh salt solution with the aid of the centrifuge until the water solutions no longer gave a reaction for protein. Indeed, the washing of the material was continued for a period of 48 hours and for many changes of salt solution after the soluble protein had disappeared from it. Then the same material was repeatedly washed with distilled water for the same length of time. The residue was now examined under the microscope and found still to have a cellular structure. A portion of it was fixed in formaldehyde and embedded in paraffin and sectioned. The solutions obtained by extraction were mixed together and tested for the presence of the globulins described by Plosz (1873) and Haliburton (1892). These solutions contained a great quantity of soluble proteins, coagulable with heat at the temperatures indicated by Haliburton. The residue when embedded in paraffin and sectioned proved to consist of small fragments of liver tissue of varying sizes, a section of one of which is shown in figure 1. An examination of this figure will show that the cells have completely retained their individuality and nothing in the preparation indicates that such a prodigious amount of soluble materials has been extracted from them. Most of the residue not fixed and embedded was immediately soluble in a N/10 solution of ammonia, but a small remnant remained of material insoluble in this reagent, which on inspection proved to be the reticular framework of the liver. In these cells so extracted there remains this structure protein upon which the integrity of the cell as a unit of structure depends (plastin of Reinke and Rodewald,

cytin of A. Schmidt). The mitochondria are little changed. The nuclear membrane is intact and some chromatin has been extracted, but much remains.

The same series of experiments were made with fresh guinea pig liver, prepared according to the method of Plosz (1873). The livers were first washed free of blood by perfusion with cold 0.85 per cent sodium chloride, injected by

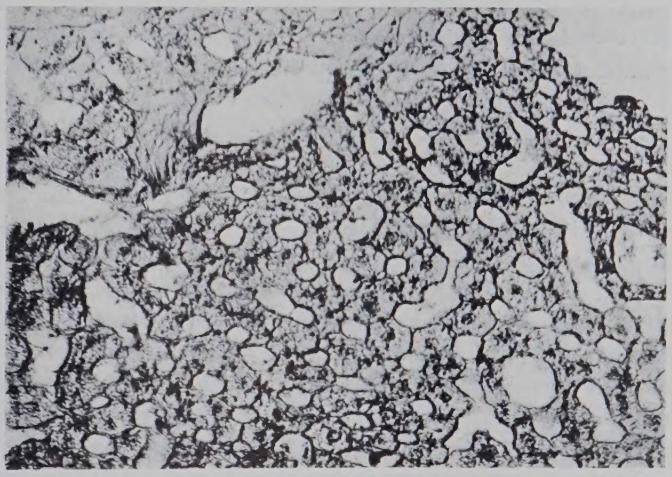

Fig. 1 Section of the liver of rabbit frozen in liquid air, dried at −30°C., extracted for 48 hours with salt solution, 48 hours with distilled water. The globulins were extracted and identified. Fixed in formaldehyde and stained with hematoxylin-eosin. Cell form and protoplasmic structure is still intact and some remains of nuclear contents may be discerned. × 400.

way of the aorta. Less than 10 minutes is sufficient under these circumstances to completely free the liver from blood. The organ is then ground up in a mortar, or minced in a latapie grinding machine or simply kneaded through bolting silk. The first and second of these methods leave more intact cells in the emulsion obtained, the third gives a larger yield of other cell products. The ground up or comminuted liver is taken up in a large volume of cold sodium chloride solution (0.85 per cent). Examined under the microscope, this emulsion con-

tains large numbers of free mitochondria, a few blood corpuscles, an enormous number of nuclei released from the cell and still greater numbers of completely isolated liver cells. Finally, there are fragments of liver tissue and shreds of connective tissue. Emulsions made in this way have been used for the separation of mitochondria as such and for the preparation of structure protein. In the first place, attention was directed to the effect of extraction of liver cells. The material sedimenting in the cold was separated from the supernatant fluid. The latter in due time after removal of suspended materials by centrifugation was tested, by acidification and boiling, for the several protein constituents recognized by Plosz and Haliburton, all of which were found in large quantities. The sediment which consisted of fragments of cells and fragments of liver tissue was washed repeatedly with cold salt solution with the aid of the centrifuge until the supernatant fluid no longer gave a test for proteins by acetic acid and ferrocyanide, by trichloracetic acid or by acidification and boiling.

At each stage in this extraction the material was examined in the following way. A small portion of the suspension was spread on a slide and examined directly. Another portion was dried in a thin smear on the slide, fixed with osmic acid and stained with anilin-acid-fuchsin and methyl green. In spite of the loss of prodigious amounts of soluble protein substances, the cells maintained their integrity as cells. The mitochondria were unaffected, except that they were slightly swollen and had a tendency to cohere into a continuous mass. The nuclei were somewhat changed, but chromatin was still recognizable in them, whether they were nuclei in intact cells or free nuclei entirely exposed to the solvent action of the solution. The nuclear membrane seemed to be a more effective barrier against the extraction of substances than the covering of the cell body.

Thus the study of the fresh liver cell confirmed the facts obtained by the study of the frozen-dried material. In each case the repeated extractions left the cell intact as a cell, but

also left the nucleus intact as a nucleus containing most of its original chromatin and with the membrane intact. The examination of the mitochondria, separated mechanically from the supernatant fluid of the primary emulsion of fresh liver cell, revealed that these, though insoluble in water and insoluble in salt solution, swelled with a slight increment of alkalinity and were completely dissolved by weak alkaline solutions. This fact provided a method for getting rid of the mitochondrial substance and of the nuclear substances and making a preparation of the structure protein in relatively pure form and studying its properties.

So far this method has been applied only to the livers of guinea pigs and rabbits and worked out in detail only for the former. Either frozen-dried material fresh from the vacuum apparatus or fresh guinea pig liver may be used. The frozen-dried material lends itself well to grinding up into a fine powder in a mortar, but since the liver cells have no connections with one another except the contacts protected by the reticular framework of the liver, the fresh liver easily releases large numbers of individual cells, so there is little to choose between the two methods. In the case of the fresh liver, however, a larger yield of mitochondria (which forms the subject of the next paper of this series) is obtained, since it is difficult in frozen-dried material to release the mitochondria from their associated cytoplasmic components. The animal is killed by bleeding and the blood immediately washed out of the blood vessels, including the liver, by perfusion with salt solution cooled to 2°C. The organ is then ground in a latapie mincing machine and rubbed through a sieve of cheese cloth to comminute it as much as possible. The material is then taken up in a large quantity of salt solution, shaken vigorously and immediately separated by centrifugation. The supernatant fluids so separated are treated separately for the preparation of mitochondria, which will be described later. The sediment of cells and masses of cells and connective tissue, etc., which also contain a large quantity of blood corpuscles which the washing has failed to remove from the

liver, is shaken up in salt solution and sedimented repeatedly until the wash water no longer shows a reaction for protein substances.

Up to this time the liver cells have retained their integrity as cells, but they still contain a considerable amount of nuclear material and practically all the mitochondria. The emulsion is then suspended in a N/200 solution of ammonia, in which the cells immediately swell and the surfaces become sticky so that the whole mass coheres into a jelly which is translucent or opaque, according to the content of free fat. At any moment, however, by vigorously shaking this gel can be broken up again into individual cells, or if the solution is neutralized or slightly acidified the substance again shrinks into an opaque mass from which the individual cells, although shrunken, may be released by breaking up on the slide with needles.

Sections of this jelly fixed and embedded and stained have lost all the mitochondria, all the nuclear chromatin, but the place occupied by the nucleus is still recognizable in the cell.

The jelly-like mass of cells which adhere together is repeatedly shaken up with salt solution containing N/200 ammonia. Large quantities of protein material, the nature of which has not yet been determined, are so extracted from it. When the salt solutions no longer give on careful neutralization a precipitate of protein substances or material coagulable by heat after acidification, the jelly is further washed repeatedly by the same concentration of ammonia in distilled water. The mechanical treatment of this suspended jelly at this stage is most important. Vigorous shaking easily captures air bubbles which float the gel and make it difficult to handle, but the material may be broken up by shaking with a rotary movement in the flask without mixing air with the solution. Under these circumstances, the gel remains broken up, but when left at rest coheres into a mass again and subsides to the bottom of the flask. Centrifugation is not necessary for the handling of the material at this stage.

If, now, to this jelly freed from supernatant fluid by decantation, a N/10 solution of ammonia be added, the jelly quickly swells into a ropy solution, and slowly but finally dissolves completely. In the intermediate stage when it constitutes a ropy solution, it resembles in its consistence the mucus from the submaxillary gland of the dog, but it slowly passes into a complete solution which is capable of being filtered. Inasmuch as the continued action of alkalies on this material results in the change of it into a substance resembling in its properties alkali albumin, it is preferable to use a stronger solution of alkali for quick solution and separation of the material from fragments of connective tissue and other debris accumulated in the process. For this purpose the jelly may be shaken up with a normal solution of sodium hydroxide which has been previously cooled. It dissolves almost instantly to form an opalescent solution from which the suspended fragments of reticulum and collagenous tissue may be quickly separated by centrifugation. This solution thus cleared from insoluble debris is immediately precipitated with acetic acid. The whole operation of solution and reprecipitation can be done in 5 minutes. The substance goes down in the form of flaky and stringy units which rapidly cohere. Under the microscope these appear as laminae with a distinct appearance of fibrillation.

The precipitate so obtained is washed repeatedly and then redissolved and reprecipitated several times. On each occasion the solution is cleared as much as possible by centrifugation. The solution remains opalescent, even if repeatedly precipitated, and it will be found later that it carries a considerable quantity of alcohol and ether soluble materials with it.

After several solutions and reprecipitations, the substance is washed repeatedly on the centrifuge with distilled water, then dried in vacuo over phosphorus pentoxide. This substance represents the residual material left in the cells of this organ after complete extraction of globulins and other soluble materials by salt solutions.

The properties of the substance from which it is derived must of necessity be studied in the original material because the alkaline treatment modifies its properties in some degree. For this purpose the cells of the Amblystoma liver are most suited. In this material the substance shows a progressive gradient of imbibition and diminution of consistence with increasing alkalinity. At the same time the cell increases in size. A similar increase of imbibition and diminution of gel consistence is seen in the acid range, so that the imbibition curve resembles the curves obtained by W. Ostwald for gelatin and R. S. Lillie for the osmotic pressure of colloids. In the material prepared by the method described above, this range of imbibition change is modified, inasmuch as after alkaline solution and reprecipitation, the substance dissolves immediately in weak alkaline solutions and resists swelling in acid solutions until a quite high acid content is reached. This material may be used for determining the chemical characteristics of the structure protein, but the properties of it in the living cell must nevertheless be investigated in the fresh frozen-dried material.

The dried material extracted in a Soxhlet apparatus successively with alcohol, ether and chloroform yields a substantial quantity of fatty substances: 0.3125 gm. yielded 0.0938 gm. of these substances, equivalent to 30.01 per cent of the dried weight of the protein. Of this 0.012 consisted of acetone insoluble materials which thus constituted 12.8 per cent of the total fat and 3.84 per cent of the original product. The substance freed from its fats gives a positive Millon reaction, though much feebler than the globulins extracted from the same cells or than the mitochondria en masse or the mitochondrial proteins from the same source. The fresh precipitate which has not been dried and which is immediately soluble in alkalies loses its solubility when suspended in water and heated to boiling, so it is denatured by heat.

It will be seen from the description of the preparation that this substance differs in its properties both in the fresh cell and in the moist preparation freed from other cell products

from the plastin of Reinke and Rodewald. Yet there is little doubt in our minds that it is substantially the same as substances seen in sections of tissue by Zacharias and Schwartz, to which the name 'plastin' also was applied. Since in the minds of most cytologists the term plastin has become a synonym for linin in the nucleus, as used by Schwartz, it seems probable that the continued use of this name for the peculiar substance in cytoplasm that we have been describing would only lead to much confusion. Accordingly, it is suggested that this substance which we have termed structure protein thus far should be called 'ellipsin,' since it is the missing substance, the lack of consideration of which invalidates previous speculations concerning the colloidal nature of the protoplasm.

It requires no argument to prove and little wisdom to perceive that this substance 'ellipsin,' which by itself maintains the cell as a unit of organic structure after the soluble globulins, mitochondria, and chromatin have been removed seriatim by solution is in reality the basis of the microscopic structure and of the organic continuity of the cell body. This is not a new idea. It was expressed by Hammersten in 1895. He said:

As far as I can conclude from my own observations and investigations, our present ideas on the albumin-like constituents of protoplasm can scarcely be correct. The chief mass of free cells and of organs rich in cells like glands consists mainly, according to my experience, not of genuine protein materials in the ordinary sense, that is not of globulins and albumins, but of far more complicated protein substances which besides nitrogen and sulphur commonly contain also phosphorus and iron. These protein substances represent the true protoplasmic protein and the albumins and globulins must be considered rather partly as nutrient material of the cell and partly as break-down products in the chemical transformation of the protoplasm.

Similar opinions have been expressed by A. Schmidt, distinguished among other things for his investigations on the coagulation of blood.

The reason for the neglect of the observations of Reinke and others in speculations on the structure of protoplasm has been the conviction that their plastin, cytin, etc., were the result of precipitation or of a process resembling coagulation. This idea was in Plosz' mind and in Haliburton's when they attempted to obtain from fresh liver, by the process used for the preparation of myosin antecedent from muscle, a similar myosin material from this organ. These efforts, however, failed. The occurrence of spontaneous gelation and liquefactions in microdissection of the protoplasm would also raise a doubt as to whether the structure protein described in these pages is a real preexistent constituent of the living protoplasm rather than a product of a gelation phenomenon occurring in an injured cell. The treatment of free-hand sections of Amblystoma liver and of the liver of guinea pig and rabbit rejects this possibility. The free-hand sections of frozen-dried material lose their soluble cytoplasmic substance with such rapidity, leaving behind a cell which is morphologically complete, although lacking these important soluble components, that the production of ellipsin by a process of coagulation or gelation is inconceivable.

Now that sufficient amounts of this material are available for chemical study, the elementary analysis and the application of the improved van Slyke method for the study of nitrogen distribution should solve all of these perplexities. This investigation is under way and will be reported later.

The relations of ellipsin to the soluble and formed constituents of protoplasm is, of course, of the greatest importance. A. P. Mathews (Physiolological Chemistry, 5th ed., 1930) has wisely observed that—

> living reactions have one other important peculiarity besides speed, and that is their orderliness. The cell is not a homogeneous mixture in which reactions take place haphazard, but it is a well ordered chemical factory with specialized reactions occurring in various parts. The orderliness of the chemical reactions is due to the cell structure, and for the phenomena of life to persist in their entirety that structure must be preserved.

R. S. Lillie (Reactivity of the Cell, in Cowdry's General Cytology) says:

> It is evident that a mere random mixture of cell constituents, in the same proportions and concentrations as in protoplasm, would not give a system having the properties of the living cell. The constituents must have a definite arrangement, and must be present in a definite physical state; only under these conditions is it possible to conceive of any kind of ordered interaction such as that underlying the life of the cell.

The investigation of the relations of structure protein to the soluble constituents of protoplasm and to the lipoids constitutes a primary responsibility of biochemical research. The globulins extractable by salt solution evidently exist in the cell in a highly dispersed state, since it is only when they are released from their prison in the structure protein that they precipitate with the reagents as they do in vitro. It is reasonable to suppose that if these globulins were continuous in the dissolved form throughout the substance of the cell as the ellipsin is continuous throughout the substance of the cell, the familiar precipitates produced by these reagents would be observed in fixed materials. This is, however, not the case unless the nature of the fixing fluid is such as to first profoundly alter the lyophilic capacity of the structure protein. It is only when we use acid solutions for the preservation of the cell that the complex of strings and granules familiar to the older students of cytology is obtained. At the present time the relations of these several things can only be dimly perceived, but since the structure protein retains its continuity when the other elements are abstracted, it is a helpful working hypothesis to regard this substance, itself a diphasic colloid, as constituting, with its associated lipoids the continuous or external phase in a polyphasic, colloidal system. The diversity of primary and secondary and tertiary, etc. phases in this system, and their arrangement in the cell body can only be guessed at, but that there is a wide chemical diversity of these secondary internal

phases, with an ordered pattern throughout any given cell, seems to be indicated from the observations made by many observers and confirmed by Bensley and Gersh ('33) on the distribution of the basophil substance of Nissl in the nerve cell and the basophil substance of Berg in the liver cells of Amblystoma. Both of these substances are distinctly segregated in special portions of the cytoplasm, and it may be postulated for purposes of further investigation that other substances and association complexes are localized in different parts of the cell body, giving that ordered arrangement which is conceded to be necessary to the orderly procedure of the vital processes.

The freezing-drying method of Altmann and Gersh seems adapted to the further elucidation of these interesting problems.

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THE ABSORPTION OF ESTROGENIC SUBSTANCES OF PREGNANT URINE ADMINISTERED ORALLY TO YOUNG RATS

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During the course of some recent investigations ½ cc. of pregnant urine was injected subcutaneously twice daily into immature female rats of the Wistar strain. The injections were begun on the twenty-second day of life. There were three females in the first litter used. From one of these the ovaries were removed on the day before the injections were begun. The ovariectomized rat and one of the normal ones were injected, while the third rat was not injected. All three rats were kept in the same cage. The injections were made about 8 hours apart, one being given in the morning and one in the afternoon. The vagina of the normal injected rat was found to be open on the morning of the fifth day injections were made. The rat was 26 days old and weighed 36 gm. The vagina of the ovariectomized injected rat was found to be open on the morning of the sixth day injections were made. This rat was 27 days old and weighed 44 gm. The fact that the vagina of this second rat opened prematurely indicates that there was enough of the estrogenic substances present in this amount of urine to cause the opening of the vagina, since prolan has been found to be ineffective in the absence of the ovaries. The third rat, which was uninjected, also had an open vagina on the sixth day injections were made. It weighed 50 gm. on the day the vagina was found open. The question then was how this last rat had been able to get the hormone or hormones which caused its vagina to open. The only possible source seemed to be the small amount of urine which it may have licked from the point of injection in the other two rats which occupied the same cage.

We, therefore, decided to repeat the experiment on another group of rats. For this purpose a litter containing four females was used. Three of these rats were kept in the same cage and treated exactly the same as the three rats of the first litter. The fourth rat was kept in a separate cage and not injected. The results in the three rats kept together were quite similar to our first results. The vagina of the injected normal rat opened on the fourth day injections were made. The rat weighed 49 gm. The vagina of the injected ovariectomized rat opened on the fifth day of injection, the animal weighing 47 gm. The vagina of the third (uninjected) rat opened on the same day as the injected ovariectomized one, this rat weighing 44 gm. The vagina of the control rat kept in a separate cage was not open on the thirty-sixth day of life when it weighed 83 gm.

Freudenberger ('32) has found that the vagina of normal Wistar rats opens at a mean age of 47 days, the range being from 36 to 66 days. Our observations in this laboratory support these findings. The vaginas of the animals in this experiment then opened very prematurely, with the exception of the one control animal which was kept in a separate cage. The latter would probably have opened at the normal time had it been allowed to live that long.

To test further the possibility of the hormones of pregnant urine being absorbed when administered orally, three rats from another litter were used. We endeavored to drop 0.3 cc. or urine into the mouths of each of these rats twice each day beginning when the rats were 21 days old. A large part of the urine ran out of the sides of the mouth. These rats were all kept in separate cages. The vaginas of all three were found open on the morning of the day they were 25 days old. The rats then had an average weight of 42 gm., ranging from 37 to 45 gm. This seemed to definitely establish the fact that there were hormones in pregnant urine which would cause the premature opening of the vagina and that they were absorbed quite well when the urine was administered by the oral route. The question then was to decide

whether the estrogenic hormones could be thus absorbed. Four rats from another litter were used for this purpose. The ovaries were removed from one when it was 21 days of age. The others were left intact. All four rats were kept in separate cages. We began dropping urine into their mouths on the following day. We attempted to give 0.3 cc. three times daily at 4-hour intervals. The vaginas of two of the unoperated animals opened on the fourth day urine was given, while the vagina of the third was found open on the morning of the fifth day. These rats had a mean weight of 40 gm. at the time the vagina was first found open, the range being from 35 to 43 gm. The vagina of the ovariectomized rats opened on the fourth day urine was administered, the rat weighing 35 gm. This seemed to definitely indicate that the estrogenic hormones as found in pregnant urine were absorbable by the oral route.

Three unoperated rats from another litter were treated according to the method just described with the same results. As stated previously, only a small part of the 0.3 cc. of urine administered each time actually stayed in the mouth. For this reason another litter was used. This litter consisted of five rats. From two of these the ovaries were removed, while the other three were not operated. We began administering urine when the rats were 22 days old, the operations having been done on the preceding day. To the first of the ovariectomized rats, 0.3 cc. of urine was administered, while to the other 0.2 cc. was given. To the first of the unoperated rats 0.3 cc. was administered; to the second, 0.2 cc.; and to the third, 0.1 cc. The urine was given thrice daily in each case. The vaginas of all five rats opened at about the same time as indicated for the experiments previously described. From this it is clear that enough of the estrogenic hormones are absorbed from 0.2 cc. urine given three times a day for 3 or 4 days to cause the premature opening of the vagina.

The urine used in the experiments thus far described was all obtained from one woman who was in the second half of pregnancy. A fresh specimen was obtained at least every other day, and the urine was kept in an electric refrigerator.

In order to make some preliminary determinations as to the concentration of the hormones in pregnant urine which caused this premature opening of the vaginas of young rats, three more litters of rats were used. In the first two litters the ovaries were not removed. The urine used was from women who were in the last month of pregnancy. Various dilutions with distilled water were made. The greatest dilution used on these first two litters was 1 part of urine to 7 parts of water. One-tenth cc. of this diluted urine was given orally three times a day at 4-hour intervals. The vaginas of all the rats opened on either the fourth or fifth day. The vagina of the rat given the most dilute urine was found open on the morning of the fifth day. It was 25 days of age and weighed 45 gm. It had received a total of 1.2 cc. of the dilute urine, this containing 0.15 cc. of the undiluted urine. The last litter used consisted of six rats. The ovaries were removed from each of them on the day they were 3 weeks of age. We began administering 0.1 cc. diluted pregnant urine three times a day at 4-hour intervals on the day following the operations. The most dilute urine given consisted of 1 part urine and 15 parts of distilled water. The second most dilute urine consisted of 1 part of urine to 7 parts water The administrations in no case were continued for a period exceeding four consecutive days. The vaginas of four of these rats opened on the fourth day. The vagina of the rat receiving the urine diluted with 7 parts of water was found open on the morning of the fifth day. The vagina of the rat receiving the urine diluted with 15 parts of water was not found open until the morning of the sixth day. It was then 27 days old and weighed 52 gm. It had received a total of 1.2 cc. of the dilute urine which contained 0.075 cc. of the undiluted urine.

DISCUSSION

Since so small an amount of pregnant urine when given orally will cause a premature opening of the vagina, it would seem logical to think that most of it is absorbed from the oral mucous membrane. The amount retained in the mouth cavity was scarcely more than the amount necessary to moisten its lining. Allen ('32) states that the "oral administration of theelin is not efficient for several investigators have shown that many times (from 20 to 100) the minimal effective subcutaneous dose is required to produce the oestrous reaction when administered by mouth." Curtis and Doisy ('31), in a preliminary report, find that "though the results are somewhat irregular, it seems that the rat unit (of theelol) enterally is from 2 to 3 times the subcutaneous unit. With respect to the establishment of the vaginal orifice theelin is about as effective enterally as it is subcutaneously, this indicating that it is absorbed in the young rat." In the report of the Council on Pharmacy and Chemistry of the American Medical Association in regard to estrogenic substances, we find in regard to the question of absorption: "This phase of the problem, despite its great importance and wide clinical use of theelin and similar preparations, is imperfectly understood. Comparatively little attention has been paid to the subject, and the data at hand are conflicting and confusing." The results of this investigation show that 0.1 cc. of pregnant urine diluted with as much as 15 parts of distilled water administered thrice daily to ovariectomized young rats will cause the premature opening of the vagina within 4 to 5 days. Perhaps, the same results would be obtained with even greater dilutions. It is hoped that this will aid in throwing more light on the problem of the absorption of estrogenic substances of pregnancy urine administered orally.

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A SIGNIFICANT EXAMPLE OF PEDUNCULATED POSTMINIMUS

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ONE FIGURE

This note is to record a case of polydactyly which forms a crucial supplement to a previous study by the writer—Spontaneous amputation of human supernumerary digits: pedunculated postminimi (Am. J. Anat., vol. 51, pp. 381–416, 1932). The present case substantiates an expressed prediction (pp. 396–397) that the diminutive rounded nodules occasionally found in characteristic postminimal sites may be merely the stalks of postminimi rather than definitive abortive digits, the digit proper in such an instance having suffered spontaneous amputation in utero. Though the prediction was warranted by the then available indirect evidences, it is gratifying to have the good fortune of securing an actual example, available for the first time in a period of some 12 years of collecting polydactyl cases.

Through the kindness of Dr. Isadore Dyer, of New Orleans, the writer received on March 16, 1934, the fetus which is the subject of report (Tulane no. 639). The left hand presented the postminimus which is to be described, this being the sole abnormality of the extremities, as well as of the fetus as a whole. After inspection of the body surface for signs of developmental defects, the viscera were carefully dissected by Dr. Richard K. Lee, of the staff of the Department of Anatomy, and no departures from the normal were discovered. The cranium was opened, the only noteworthy finding being an extensive subdural hemorrhage with ex-

tension into the choroid plexus of the third ventricle. Further points concerning the fetus are here enumerated: negro; female; CR length, 25 cm.; foot length, 5.5 cm.; born living; second pregnancy of the 17-year-old mother, both terminated as miscarriages; fetal age slightly less than 6 calendar months, as indicated by menstrual history and level of uterus just prior to miscarriage.

The status of the new case may be best appreciated by referring first to the results of the study just cited. Evidences were presented to show that postminimi may suffer arrests in differentiation as well as growth, the extreme effects being displayed in the pedunculated forms. These arrests are identified with the existence of 'focal deficiency,' as Streeter terms the "greatly decreased vitality and defective growth capacity" which may exist locally in members otherwise normal, sometimes leading to amputation. Whether through severe focal deficiency or by mechanical handicaps incident to the state of pedunculation, an occasional postminimus may be spontaneously 'amputated,' or sloughed. Among the fifty-six individuals bearing postminimi treated in that account, three were reported as suggesting amputation in progress: an infant 8 weeks of age (Tulane no. 409) and two late fetuses in the Carnegie Collection (sitting height 314 mm., no. 4526–19, illustrated in figure 11; sitting height 275 mm., no. 3545). One case (a fetus of 80 mm. sitting height, Carnegie no. 652 e, illustrated in fig. 12) was shown to have a postminimus attached by "a pedicle so thin and delicate that amputation would seem inevitable, either through mechanical interference with circulation or trauma to the pedicle." Two cases were interpreted tentatively as amputations which had been completed in utero, and since these have so immediate a bearing on the present instance, the comments concerning them are quoted in full.

> The first is [Tulane no.] 414, a male infant aged three days, whose left postminimus is . . . . . [typical of the narrowly pedunculated forms]. It is the right hand which forms the object of present interest. At the level of the middle phalanx of the right minimus there is a hemispherical elevation 2 mm.

in diameter and of about the same height. The summit of the elevation bears a minute point of apparent exaggerated cornification. The occurrence of this hillock in an individual having a postminimus on the opposite hand in a corresponding position clearly points to its identity as an expression of polydactyly, yet there is no means of establishing positively the alternative possibilities as to its specific nature. The hillock may represent the total but abortive formation of a postminimus and on the contrary, there is perhaps greater likelihood that it is the stump remaining after spontaneous amputation of a postminimus equivalent to that found on the other hand. The latter alternative is supported by the occurence of apparent active amputative phenomena in the several cases preceding. A further observation in this same connection is furnished in a fetus in the Carnegie collection (C4110-8, female, sitting height 303 mm.), where evidence is present to support the interpretation that a similar hillock is an amputation stump. In this specimen both feet have well-developed postminimi. The right hand is normal, while the left one bears a nodular elevation at the base of the minimus (figs. 13 and 14). This nodule, again, may represent an abortive postminimus, though its nature as an amputation stump is suggested by the epidermal surface markings. While the nodule is situated in an area marked by the characteristic epidermal ridges of the volar surface, and indeed the nodule itself shows such ridges over its distal and proximal portions, its summit shows no ridged configuration. Since ridge-forming capacities are present in the epidermis of the marginal portions of the nodule, it is difficult to explain their lack in the summit region except as the correlate of repair. If the nodule were originally developed in its present form, and thus being simply an abortive digital structure, ridge formation would be expected over its entire surface, as would be true also if an amputation occurred prior to the period of ridge differentiation, followed by reorganization of the stump and normal ridge differentiation on its surface.

The findings in this group of cases indicate the need for caution in ascribing a specific identity to the diminutive nodular 'digits' as expressions of polydactyly. It cannot be denied that we have only indirect evidence that the nodules in T414 and C4110-8 are amputation stumps, and, moreover, that cases do occur (Streeter, '30, pp. 14-18) in which one or more digits of the normal series are suppressed in various degrees, sometimes appearing as mere nodules. In a polydactyl previously described (Cummins, '26, case

23) there is a small rounded mound near the base of the fibularmost digit in a seven-toed foot. Originally referred to as 'possibly representing an abortive digit,' I would now mention the alternative possibility that it may be an amputation stump, the amputation, if such it is, having occurred prior to ridge differentiation as evidenced by the dermatoglyphics. A case of postminimal nodule (abortive digit or amputation stump?) is illustrated by de Lima ('27, fig. 15), and I have a report by a reliable observer of a familial polydactyly in which the supernumerary digit is consistently represented by a wart-like mound at the base of the minimus of the hand. We cannot be sure that these cases represent postminimi spontaneously amputated in utero, but at the same time it is unwise to make a positive assertion that such nodules are definitive abortive digits.

The new case represents the probable state of the postminimi in the subjects just described, for the period preceding the sloughing of the digit. To describe its gross features is unnecessary, since the photograph (see figure) illustrates the essentials: a rounded hillock, which is the surviving part of the postminimal stalk, corresponding to the nodule of the last two cases; the remnant of the more distal part of the stalk, reduced to a delicate thread; the digit proper, a tiny ovoid bulb of darkened tissue.

The digit was excised for serial sectioning, a large block including the area of attachment being taken with it. The basal nodule presents normal histological structure throughout both epidermis and deeper tissues, but at the very beginning of the thread-like stalk there is an abrupt transition into abnormal structure, which characterizes the stalk and ovoid digital mass. The epidermis lacks papillae and glands; it is thinned, and extensively hyalinized, the cells being flattened parallel with the surface and presenting pycnotic nuclei. Degenerative signs are marked in the deep tissues. Pycnosis and karyorrhexis are general through the connective tissue, and the fiber bundles show beginning dissolution; a vestige of degenerated cartilage appears in the interior. No persistent blood vessels are to be found within the stalk or digital mass, though there are numerous cells laden with granules of blood pigment. It is evident, there-

fore, that the digit is passively undergoing degeneration; in its present state there is neither congestion nor inflammatory reaction. While the fetus was obtained very soon after death and the material promptly fixed, it should be further emphasized that none of the observed changes is due to delay in fixation, since this possibility has been ruled out by comparing the normal tissues, excised with the degenerating digit.

Fig. 1 Hand photographed after formalin fixation. × 9/5.

It is quite evident, from the foregoing observations, that only the basal nodule is destined to persist. Were the nodule observed after complete dissolution of the digit, it would appear as in the two examples referred to above, perhaps with "a minute point of apparent exaggerated cornification," as in T414, representing a vestige of the thread-like stalk. The case therefore demonstrates that a postminimal nodule may be merely the stalk of a postminimus rather than a definitive, abortive digit.

ON THE CONTROL OF REPRODUCTIVE ACTIVITY IN AN ANNUAL-BREEDING MAMMAL (CITELLUS TRIDECEMLINEATUS)[1]

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Reproduction is fundamentally rhythmical in character, since the reproductive system, instead of being a continuously functioning system, becomes active at recurring periods. In vertebrate animals, in general, these rhythms recur but once each year, the peak usually coinciding with the months of spring. The most conspicuous exceptions occur among domesticated birds, and among mammals where the basic cycle—at least in the female—may be crowded into a few days or weeks and repeated at regular intervals many times during the year; usually the male of such forms remains in a continuous reproductive state and may mate at any time with a receptive female.

The mechanism controlling such exact periodicity has long been of interest to the general biologist and to the clinician who is seeking a basis for treatment of reproductive disorders in the human species. Observations in this laboratory on the effects of administering gonad hormones and other substances to such laboratory mammals as the rat and guinea pig suggested a mechanism involving a reciprocal relationship between the gonads and the anterior pituitary gland. A working hypothesis, which has proved of much value in our experimental work, was developed by Moore and Price ('30, '32) and by Moore ('31). A great many basic facts con-

[1] This investigation has been aided by a grant from the Committee for Research in Problems of Sex of the National Research Council; grant administered by Prof. F. R. Lillie.

tributed from other laboratories during the past 10 years have fitted into the basic ideas involved and permitted their extension to many other phases of reproduction; the factual basis for the concept is discussed at some length by Moore and Price ('32). About the same time an almost identical hypothesis for oestrous cycle control was suggested by Brouha and Simonnet ('31).

In the development of the reciprocal interrelationship hypothesis, reference was made to the need for careful analyses of reproductive activities in seasonal breeding types of mammals where the fundamental pattern still exists. This formed the basis for a project begun in 1931, under the general guidance of Moore, to investigate normal events throughout the year in both sexes of an annual-breeding species and to attempt a further analysis of such events by experimental procedures. The program included a study of the female system by Simmons and the male system by Wells—involving gonads and all accessories in each sex—while tissues recovered from these animals were used for a cytological study of the pituitary by Nelson, the adrenals and thyroids by Zalesky, and the teeth (especially as indicators of calcium metabolism) by Isaac Schour. It was hoped by such a program to throw light on rhythmical control of reproduction as well as on the general participation of the endocrine glands in this process.

The animal chosen for this study was the thirteen-lined ground squirrel (Citellus tridecemlineatus), so common in the Chicago region that more than a thousand animals have been captured afield and brought into the laboratory for study and experimentation. They have been available in the laboratory every month in the year, and fresh from the field between April and November; they have been planted in pens in the country and in outdoor pens and cages at The University of Chicago, where they dug underground in their natural habitat and hibernated in normal fashion, making it possible to dig animals from burrows at any time during the winter hibernation period. Certain phases of the activity of the reproductive system of this form have recently received attention by

Johnson and his students ('31, '33) at Kansas, and by Mossman and his students ('32) at Wisconsin. The principal results of our study on these animals may now be outlined.

Female ground squirrels emerge from hibernation during the early warm days of spring, usually about the third week in April, some 10 days later than the males. A single period of oestrum occurs, lasting only until coitus a few days after emergence—though oestrum in segregated females continues for several weeks. Gestation occupies 4 weeks, and litters are delivered from mid-May to the first week in June. The reproductive tract undergoes the greater part of its involution immediately after parturition, though during the 4 to 6 weeks of lactation the ovaries actually increase in size; after weaning, the ovaries and all accessories gradually regress to a low point, and remain essentially inactive during summer and fall. About January 1st, while females are in mid-hibernation, the ovaries begin to show signs of activity, followed by a slow gradual development of oviducts, uterus, vagina and mammary glands toward their peak in spring; older animals emerge in a more advanced state of reproductive activity than do those born the previous spring.

Males usually appear above ground during the first 10 days of April, when some are in a mating condition. Testes are large and scrotal, and contain spermatozoa, while reproductive accessories are approaching the peak of functional maximum, reached in most males by late April. Usually by July 1st the testes have declined in size and activity and have receded into the abdominal cavity, and spermatozoa have disappeared from the epididymis; all accessories—including epididymis, vas deferens, seminal vesicles, prostate, Cowper's and bulbar glands, etc.—have undergone regression, and remain more or less quiescent during the first part of hibernation, which begins in October. Spermatogenesis gradually becomes evident in December, with spermatozoa appearing in seminiferous tubules in January or February. The testes are essentially aspermatic from July to January, with some variations depending on the age of the animal.

Various types of experimentation have been employed to analyze the factors controlling this more or less exact succession of events in reproductive activity, only a few details of which can be mentioned here.

Despite the involuted state of accessory reproductive organs during the period of inactivity, it has been found in both sexes that gonad removal induces a slightly greater state of involution. Each gonad, therefore, exerts at least a minimal influence on accessories at such time. Unilateral gonadectomy induces no detectable changes in the accessories, though it results in slight compensatory hypertrophy of the remaining gonad in the male.

The ovary and female accessories are capable of responding to stimulation at any time of the year. They will respond to fresh anterior pituitary implants, untreated urine from pregnant women and such urinary extracts as antuitrin-S and hebin, and also extracts of both fresh and dried sheep pituitary. Development of the ovary, oviduct, uterus, vaginal tract (with introitus and full oestral smear), and mammary glands can thus be induced at will by application of such gonad-stimulating substances. A similar development of accessories can be induced in spayed females by the introduction of such oestrogenic substances as oestrin and theelin, which do not appreciably affect the ovary itself during its low period of activity.

The testis of all male accessories can be stimulated to a high degree of activity at any time of the year by the application of gonadotropic substances such as fresh anterior pituitaries, extracts of dried sheep pituitary, human pregnancy urine and antuitrin-S. All such treatments stimulate the testis to produce and release male hormone, which in turn stimulates the accessories to a complete functional condition. Fresh pituitary, human pregnancy urine and antuitrin-S have also induced spermatogenesis, with at least stimulation of residual cells present to go through intervening stages and metamorphose into spermatozoa. Injections of bull testis extract bring all male accessories to a high state of activity at any time of the year, in both normal and castrated animals.

Oestrin injections have caused gross-weight increase in accessories of normal and castrated males. Microscopical study has shown, however, that this is a peculiar response of connective tissue or muscle, not a normal secretory response of the epithelium of these glands. Oestrin has constantly produced injuries to the testes at their high period of activity, with little effect on those at the low point.

Extracts of bull testis (Gallagher-Koch) caused opening of the vagina in female ground squirrels, with perceptible stimulation of the female reproductive tract to the size of that of average young females in mid-April. The extract apparently contained traces of oestrin, though not enough to induce normal secretory activity or show even an approach to prooestrum. As in males given oestrin, females given extract of bull testis showed largely a peculiar response of connective tissue or muscle.

Thus it was shown that the gonads and reproductive accessories of both sexes are capable of responding to proper stimulants at any time of the year. Gonad stimulants, showing no effects on accessories of gonadectomized animals, stimulate the gonads themselves to secretory activity; and the gonads in turn supply the sex-specific hormone that stimulates accessories. Hence the extended period of inactivity in this annual-breeding type of mammal is merely due to the absence of the agent necessary to induce activity in the gonad of each sex. Since it became apparent that, as in many laboratory and domesticated animals, anterior pituitary material is a gonad-stimulating agent for the ground squirrel, we have investigated the question of seasonal activity in the anterior pituitary of this animal.

Using the immature female rat as a test animal, we find that the anterior pituitaries of normal ground squirrels from the field on approach of or during active reproduction—males with scrotal testes, and prooestral or oestral as well as pregnant females—contain a high amount of gonad-stimulating substances. Anterior pituitaries from animals killed during the long period of sexual inactivity—males with abdominal

testes, and females with small and inactive ovaries—showed no physiologically detectable trace of gonad-stimulatng hormone. These findings are supported in the main by results of similar tests made by injecting ground squirrel blood serum into immature female rats.

Castration has induced increased potency in anterior pituitaries of male and female ground squirrels, even during the low period. Immature rats receiving anterior pituitaries from castrated or spayed ground squirrels had ovaries two to seven times heavier than those receiving glands from unoperated ground squirrels at the same season of the year. Blood-serum tests verified these findings. These results demonstrate that even at the low stage of their activity the gonads exert some inhibiting influence on the anterior pituitary, the low production of gonad hormone being indicated by the low grade of activity of the reproductive accessories.

Pituitaries of both sexes show a seasonal variation in weight and cytological picture correlated with such changes in hormonal content. With the onset of sexual activity there is progressive increase in weight, which remains relatively constant throughout summer, decreasing slowly to a low point in December and January. This variation appears to be confined to the anterior lobe.

The usual cell types are present in the anterior pituitary of the ground squirrel. Cell counts show that basophiles increase in number and size during the breeding season. Their granules are large and numerous at such time, but during summer they undergo progressive degranulation and finally appear to dedifferentiate to a chromophobic condition. Coincident with recurrence of changes in the reproductive organs, the basophiles reassume their granular condition. Sex differences in cell population of the anterior lobe, found in certain short-cycle forms, do not appear to be present in the ground squirrel. Gonadectomy induces a basophilic increase which differs somewhat from that occurring at the onset of the breeding season. Administration of gonad hormone preparations prevents or abolishes the basophilic in-

crease caused by removal of the gonads. Direct administration of gonad hormone in large amounts—or its production within the animal by prolonged administration of gonad-stimulating preparations—causes a degranulation of the basophiles—a reaction which has been previously observed in the rat.

Our investigation failed to reveal any direct or otherwise significant correlation between the sexual cycle and the thyroid gland. The latter follows its distinct, independent annual rhythm, with low activity during the hibernating season and high activity from April to October, apparently associated with cyclic fluctuations in general metabolism rather than with reproductive activity. Results already obtained and experiments now in progress seem to suggest that thyroidectomy does not modify the sexual cycle in either the male or the female ground squirrel. It seems, therefore, that the annual sexual cycle on the one hand and seasonal changes in the thyroid on the other, are conditioned and regulated by different hormonal or neuro-hormonal complexes, in each of which we have shown the anterior pituitary to be involved.

Regarding the adrenal gland, indications point to a definite interrelationship between the adrenal cortex and the reproductive cycle. Thus, a significant increase in adrenal size and weight, traceable to cortical hypertrophy, occurs in both male and female ground squirrels during the breeding season or as a result of artificial stimulation of gonads and reproductive accessories during the period of sexual inactivity. Histologically, this cortical hypertrophy is associated mainly with expansion of the reticular zone and the differentiation, within the latter, of a characteristic, highly-developed, outer sub-zone. Bilateral castration, in either sex, leads to degenerative changes in the adrenal reticularis as well as to its dedifferentiation. Certain aspects of this complex adrenal-gonad relationship call for further clarification.

These many correlations indicate strongly that, as in other animals, the anterior pituitary of the ground squirrel is the regulator of reproduction. A low state of anterior pituitary

activity is accompanied by a change in its cell constituents and by low reproductive activity; pituitary activity increases on approach to and during high reproductive activity; the addition of anterior pituitary substances stimulates the reproductive system. All this builds up a correlation difficult to visualize in any other manner than as a reciprocal relationship. Just what part the adrenal plays in this complex is as yet uncertain; but the thyroid apparently does not play an important role, at least in the form under consideration.

The question then remains, what governs the activity of the anterior pituitary? The solution of this question appears to be the key to the control of seasonal reproductive activity. It is obvious that some environmental factor—perhaps differing in the different species—is operative in the seasonal control of annual-breeding types, playing a major role in regulating the pituitary, which in turn induces reproductive activity. The experiments of Rowan ('25–'30), Bissonnette ('30–'32), Baker and Ranson ('33), and Hill and Parkes ('33) have emphasized the importance of light as a conditioning factor in the chain of responses; while those of Craig-Bennett ('31) on fishes emphasize the absence of a light effect, but show the importance of relative changes in temperature. Deductively, one would tend to exclude light as a possible factor in the control of these events in the ground squirrel, for the reason that reproductive activity is well started underground in the darkness of the winter burrow. Our experiments with the daily addition of light by means of electric lights—gradually lengthening daily light periods from 12 to 22 hours within a period of 2 months—have failed to suggest any stimulation. Further continued subjection to light periods of 22 hours a day for 3 months also failed to produce observable effects.

Other experiments with diet, darkness, constant warm or cool temperatures, removal from brief periods of cold or hibernation into warmth, and similar manipulations, have been essentially negative in efforts to induce development of the reproductive system at seasons when it is unusual for such

development to occur in the ground squirrel. Suggestive results have been obtained in the female, however, with constant cold temperatures. Females maintained at ordinary room temperatures without hibernation usually show little or no tendency to reproductive activity; yet others kept continually for several months in cold and darkness, with more or less normal hibernation, exhibited sexual development at any time of the year, and periods of oestrum have thus been induced and maintained for many months at times of the year when the animals are ordinarily sexually inactive. On the other hand, males in the laboratory in the absence of low temperature and hibernation continue the course of events typical for them in field hibernation; non-hibernating males reach a breeding condition earlier than those in which hibernation occurs.

Our observations and experiments suggest that the difference between reproduction in annual breeders on the one hand and laboratory and domesticated animals on the other is due to a differential activity on the part of the anterior pituitary. Instead of remaining in continuous secretory activity as in constant and short cycle breeders, this gland—with its apparent long annual cycle, controlled to some extent by environmental factors—is periodically active and inactive, and is the real ruler of the annual reproductive rhythm. Many additional points will be treated in detail in later publications and several unsettled points then discussed for the bearing they have on such an attempted explanation.

Many parts of the experimental phase of this study were possible only through the kindness and courtesy of others in generously providing hormone preparations. We gratefully acknowledge our indebtedness to the following individuals for their cooperation during these experiments: Prof. H. B. Van Dyke and Zonja Wallen-Lawrence, for samples of pituitary and urinary hebin; Dr. Oliver Kamm and Parke, Davis and Company, for the preparations of whole sheep pituitary gland extract, antuitrin-S and theelin; Prof. F. C.

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A STUDY OF THE SEASONAL CHANGES IN THE ADRENAL GLAND OF THE THIRTEEN-LINED GROUND SQUIRREL (CITELLUS TRIDECEMLINEATUS), WITH PARTICULAR REFERENCE TO ITS SEXUAL CYCLE [1]

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ONE CHART AND TWO PLATES (SEVEN FIGURES)

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INTRODUCTION

The literature dealing with endocrine interaction in general and with adrenal-gonad interrelationships in particular presents a bewildering confusion of disharmonious findings. Thus, a casual glance through the tabular summaries included in chapter XVII of 'Sex and Internal Secretions'[2] will suffice to impress one with the inconsistence of the results obtained thus far in the investigation of the effects of bi-

[1] This investigation has been aided by a grant from the Committee on Research in Problems of Sex of the National Research Council; grant administered by Prof. F. R. Lillie.

[2] This chapter is a contribution of Severinghaus, Engle and Smith to the volume named above, and is listed in 'Literature cited' under the authors' names.

lateral adrenalectomy upon the reproductive system. As for the effect of administration of adreno-cortical substance on the gonads, the corresponding reports are likewise extremely contradictory. Eaton et al ('29) find an increase in the weight of testes of young chicks fed with desiccated adrenal cortex. Chidester, Eaton and Thompson ('29) conclude that young female rats fed 0.5 gm. of desiccated adrenal cortex daily reach sexual maturity sooner than untreated controls or littermate females fed 0.5 gm. desiccated adrenal medulla daily. Engelhart ('30), using a lipoid cortical extract, obtained in young female rabbits a marked hypertrophy of the uterine mucosa and musculature. Müller ('31), however, injected immature female rats with a protein-free extract of adrenal cortex of beef and found that the experimental animals came into puberty considerably later than the controls and that the entire reproductive tract of the former was markedly atrophied. The same extract used by Klein ('31) on young male rats stimulated greatly their testes and reproductive accessories and induced precocious sexual development. Corey and Britton ('31) reported precocious maturation of the gonads of young albino rats following injection of a cortico-adrenal extract prepared by a modified Swingle-Pfiffner method. According to Corey and Britton, females responded to the treatment more effectively than males. Gaunt and Parkins ('33), however, working on rats and chicks, were unable to confirm these observations. Atwell ('32) injected hypophysectomized tadpoles of Rana sylvatica with Hartman's cortin, with the result that the ovaries of the treated animals averaged one and one-half and two times the size of ovaries from untreated hypophysectomized controls. This result suggests that the adrenal cortical hormone exercises a stimulating action on the ovary directly or, at least, not through the intermediary of the hypophysis.

In view of these and many other conflicting and indefinite results, it was decided in this laboratory to attack the problem of the adrenal-gonad interrelationship from an entirely different, obviously promising, though hitherto overlooked angle.

The assumption underlying our decision was that any significant interaction which might exist between the adrenal gland and the gonads ought to be demonstrable seasonally in an annually breeding wild mammal. It was, furthermore, realized that this type of test for the adrenal-gonad interrelationship would evidently be more critical, more fundamental, and more conclusive than experimental procedures involving administration of extracts of unknown chemical composition and, therefore, of doubtful purity, or removal of a gland so essential to life that its extirpation profoundly and complicatingly upsets the entire organism.

Our choice for this seasonal study fell upon the thirteen-lined ground squirrel (Citellus tridecemlineatus), an annually breeding mammal which offers the additional advantages of convenient size, fairly good tolerance of captivity, and relative abundance in the Chicago region.

It was anticipated that the seasonal changes in the endocrine glands of this animal would furnish the desired basic approach to the problem of interglandular relationships. Accordingly, the seasonal variations in the adrenal gland of the thirteen-lined ground squirrel constitute the chief object of this study; in addition, however, an experimental analysis of some of the relationships involved was attempted, and its results were likewise included in this report.

The present investigation constitutes a part of a cooperative study of the physiology of reproduction in relation to the endocrine glands. A summary of the results of this general project is presented elsewhere (Moore, Simmons, Wells, Zalesky and Nelson, '34). The project was initiated and directed by Prof. Carl R. Moore, for whose kind and instructive guidance throughout the course of this work the writer is profoundly grateful. The reproductive cycle and its experimental modification in the female were studied by Mr. Geo. F. Simmons; those in the male, by Mr. L. J. Wells, and the hypophysis of both sexes with regard to the sexual phenomena under investigation, by Dr. W. O. Nelson. Dur-

ing the experimental phases of this study a great many hormone preparations have been utilized. It is, then, with great pleasure that the writer makes the following acknowledgments: to Dr. H. B. Van Dyke and Mrs. Z. Wallen-Lawrence, of the Department of Physiological Chemistry and Pharmacology, The University of Chicago, for pituitary hebin extracted from dried sheep pituitary powder, and to Dr. T. F. Gallagher and Prof. F. C. Koch, of the same department, for testis hormone (bull testis extract); to Dr. R. G. Gustavson, of the University of Denver, for oestrin (placental and pregnancy urine extracts); and to Dr. Oliver Kamm, of Parke, Davis & Co., for antuitrin S and whole sheep pituitary suspension, kindly furnished through the courtesy of Dr. W. O. Nelson. Untreated human pregnancy urine, collected locally, was likewise used.

The writer's sincere thanks are also due to Mr. Geo. F. Simmons and Mr. L. J. Wells for their splendid, hearty cooperation and invaluable general assistance; and to Dr. Normand Hoerr and Dr. W. O. Nelson, for their helpful suggestions regarding various technical procedures employed in this study.

MATERIAL AND METHODS

The glands treated in this investigation were removed at autopsy from animals used by Mr. Simmons and Mr. Wells in their study of the sexual aspect of the general project. Since their reports, to be published in the near future, include a detailed description of the conditions under which the laboratory confined animals were kept, these particulars have been omitted from the present paper. Most of the seasonal work, however, was done on animals killed shortly following their capture.

The weighing of the glands was accomplished by means of an analytical balance. The tissue—one adrenal from each animal sacrificed[3]—was weighed after fixation in Bouin's fluid while submerged in the latter.

[3] The bilateral differences in the weight of the glands were generally found to be rather insignificant.

The fixing agents employed were chiefly Bouin's fluid and formalin-Zenker, the latter having been prepared according to the formula used by Hoerr ('31). Whereas Bouin fixation gave dependable results for general histological structure and relationships, the finer cytological details were brought out more satisfactorily in the formalin-Zenker-fixed material. Paraffin sections were obtained from 3 to 5 μ in thickness. The staining was done chiefly with Krichesky's ('31) modification of the Mallory connective tissue stain, which proved excellent for the adrenal; hematoxylin (Harris' modification) with eosin counterstaining was also used.

The method found to be most effective and reliable for the preservation and staining of the adrenal lipoids involved fixation in Regaud's fluid and staining in Romeis' ('29) sudan orange. This valuable method was suggested to the writer by Dr. N. Hoerr and is described fully in one of the latter's forthcoming publications.

The Reese ('31) modification of the Bell ('29) osmic method for lipoids also gave good, though less dependable results.

GENERAL HISTOLOGY OF THE GLAND

The adrenal gland of the thirteen-lined ground squirrel presents several peculiarities, and, therefore, a more or less detailed description of its general histology is deemed desirable.

The outer investment of the gland consists of a fibrous capsule of varying thickness. The zona glomerulosa is usually narrow, and has never been observed to exceed one-sixth of the total width of the adrenal cortex. Its cells are normally closely packed within the spheroidal glomeruli, with apparently little interglomerular connective tissue. The fibrous radial trabeculae dividing the glomeruli are, however, conspicuous, and often extend into the zona fasciculata.

The typical glomerulosa cell has a small, spherical, centrally placed and densely chromatined nucleus; a limited amount of cytoplasm which, in glands prepared by ordinary histological methods, is more or less vacuolated; and usually

indistinct cell walls. The cytoplasmic vacuolization, from comparisons made with Romeis lipoid preserving preparations, appears to be due to the dissolution of lipoids by the acetic acid fixatives, alcohols, and high temperature paraffine infiltration employed in the histological routine. Usually the inner half of the glomerulosa is more intensely vacuolated.

The zona fasciculata of the adrenal cortex is highly variable in thickness and is often transitional in character, gradually blending the zona glomerulosa into the reticularis and making the zone limits within the cortex extremely indistinct. Whereas in other mammals (e.g., man, dog) the fasciculata is the widest, best developed zone, in the thirteen-lined ground squirrel it is usually narrow and inconspicuous, and, under certain conditions specified below, it cannot be definitely identified. Whenever present and clear-cut, the fasciculate zone answers the description of the 'spongiosa' of authors. It consists of large cells attaining up to 35 μ in diameter, spherical or polyhedral in shape, with a considerable amount of flocculently granular, more or less intensely vacuolated cytoplasm, and a large, round, vesicular nucleus containing a loose chromatin network and usually showing one to two nucleoli. Also here the vacuolization is traceable to lipoids dissolved by ordinary histological technique. The fasciculata cells are arranged in a fashion typical of this zone in all mammalian adrenals, namely, in cords radially disposed and separated from each other by connective tissue and narrow sinusoids.

The most outstanding layer of the adrenal cortex, in the animal under consideration, is the reticular. In adult animals it usually represents the widest cortical zone, highly conspicuous by the abundance of dense, deeply staining material (pigment?) in the cytoplasm.

The typical reticularis cells are smaller than the fasciculata cells, are spheroidal or cuboidal in shape, and their nuclei resemble those of fasciculata cells. The arrangement of cells in the typical reticularis is also identical with that observed in the corresponding layer of other mammalian adrenals: the

cell cords here anastomose in an irregular fashion and are again divided by stroma tissue and blood sinuses.

The adrenal medulla is variously shaped and variously located within the gland—a situation anticipated from the embryology of the adrenal chromaffine system. Accessory medullary nodules are often found embedded in the cortex. The main medullary mass may be eccentric and even reach the periphery of the gland, particularly at the hilus. Such medullary outgrowths into the cortex usually disturb the zonation in the adjacent cortical areas. The differentiation of reticularis cells into the so-called 'dark' and 'light' cells described, for the guinea pig, in detail by Hoerr ('31) and occasionally encountered in the adrenal of Citellus tridecemlineatus, is invariably more pronounced in the vicinity of the medullary 'peninsulae.'

The medulla consists of variously shaped and irregularly arranged cell masses separated by fibrous connective tissue and by large sinuses in which one finds abundant erythrocytes, debris of destroyed cortical cells, and a substance staining a homogeneous pale blue with Mallory, probably a coagulum of blood plasma. A medullary cell is about the size of a fasciculata cell, elongated, more or less distinctly cuboidal or somewhat triangular in shape, with a vesicular nucleus showing one to four nucleoli. The nucleus is usually centrally located within the cell; it may, however, be shifted toward either pole of the long cellular axis. The immediate proximity of a blood sinus does not seem to influence the position of the nucleus within the cell. The cytoplasm of the medullary cell contains extremely fine granules staining light purple with Mallory. Whether this granulation is of any significance in the elaboration of the chromophilic material is unknown. Islets composed of cortical cells, most closely resembling those of the zona reticularis, are also found here and there among the cords of medullary cells. Occasionally one is fortunate enough to obtain a section through the medulla at a level containing an unusually large sympathetic ganglion which may show as many as some twenty relatively huge, irregularly shaped cells with a deeply stain-

ing cytoplasm and a good-sized nucleus. Sympathetic ganglia may rarely be extra-medullary and even extra-capsular in location.

Small accessory adrenals consisting exclusively of cortical tissue are not infrequently found in the vicinity of the main gland. Whether accessory adrenals may also be located in other regions of the abdominal cavity, was not ascertained.

The distribution of lipoids in the normal adrenal is as follows: there is usually a very narrow peripheral layer poor in lipoids, or entirely devoid of them. The cytoplasm of the inner glomerulosa and particularly of the entire fasciculata is packed with varying large spherical droplets of lipoid material which take sudan orange most intensely. The lipoid content of the reticular zone and of the cortical islets of the medulla is low and probably finely divided and homogeneously distributed throughout the cytoplasm. The medulla proper does not take the stain at all and is thus devoid of lipoids and fats which have an affinity for sudan orange. Occasionally large spherules of lipoid material are seen scattered throughout the inner reticularis, in close proximity to the medulla. They are probably associated with focal degeneration which is most pronounced at this level.

A systematic destruction of presumably senescent cortical cells is carried on in the inner reticularis, in close proximity to the medulla. This innermost layer of the adrenal cortex often shows a ring of connective tissue fibers entangling cells in various stages of degeneration; hyperchromatic, pycnotic, and disintegrating nuclei; and debris of all sorts. The adjacent medullary sinuses likewise represent a veritable 'dumping ground' for cellular remains apparently derived from the cortex and eventually removed by the blood stream.

Mitotic figures are exceedingly rare in histological preparations of Citellus adrenals. In spite of a most thorough search, the writer found only two instances of mitotic division among some 600 adrenals examined. The two mitoses observed were in the glomerular zone. Thus, regeneration in the adrenal gland could not be directly observed. The work of other students of the mammalian adrenal, notably of

Graham ('16) and Hoerr ('31) offers, however, satisfactory evidence to the effect that cells proliferate in the peripheral layers of the adrenal cortex and migrate inward to replenish the losses sustained at the medullary border.

SEASONAL VARIATIONS IN THE ADRENAL OF CITELLUS TRIDECEMLINEATUS

The following quotation from the report on the general project of which this study is a part (Moore, Simmons, Wells, Zalesky and Nelson, '34) will familiarize the reader with the essential features of the sexual cycle in Citellus tridecemlineatus:

. . . . Female ground squirrels emerge from hibernation during the early days of spring, usually about the third week in April, some ten days later than the males. A single period of oestrum occurs, lasting only until coitus a few days after emergence . . . . Gestation occupies four weeks, and litters are delivered from mid-May to the first week in June. The reproductive tract undergoes the greater part of its involution immediately after parturition, though during the four to six weeks of lactation the ovaries actually increase in size; after weaning, the ovaries and all accessories gradually regress to a low point, and remain essentially inactive during summer and fall. About January 1, while females are in mid hibernation, the ovaries begin to show signs of activity, followed by a slow gradual development of oviducts, uterus, vagina, and mammary glands toward their peak in spring. . . .

. . . . Males usually appear above ground during the first 10 days of April, when some are in mating condition. Testes are large and scrotal, and contain spermatozoa, while reproductive accessories are approaching the peak of functional maximum reached in most males by late April. Usually by July 1 the testes have declined in size and activity and have receded into the abdominal cavity, and spermatozoa have disappeared from the epididymis; all accessories, including epididymis, vas deferens, seminal vesicles, prostate, Cowper's and bulbar glands, etc., have undergone regression, and remain more or less quiescent during the first part of hibernation, which begins in October. Spermatogenesis gradually becomes evident in December, with spermatozoa appearing in seminiferous tubules in January or February. The testes are essentially aspermatic from July to January. . . .

The adrenal cortex of the thirteen-lined ground squirrel exhibits seasonal changes which can be definitely correlated with the animal's annual sexual cycle. All histological method employed in the course of the present investigation failed, however, to disclose any seasonal variations in the adrenal medulla.

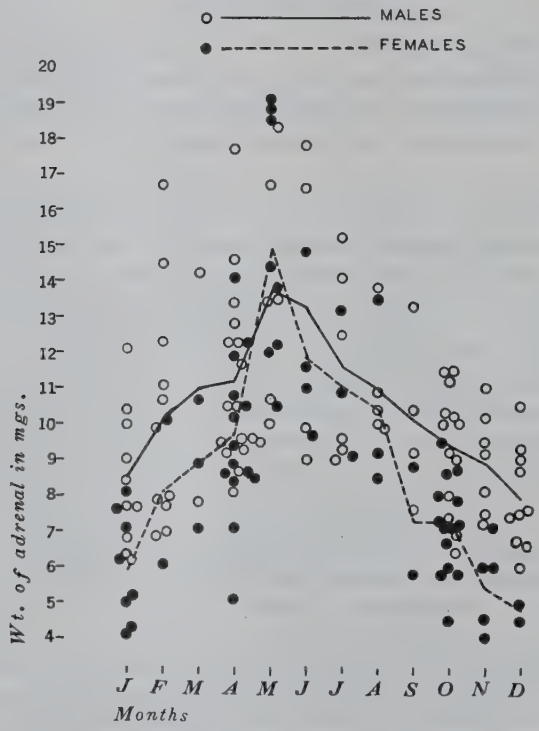

Chart 1 Seasonal fluctuations in the weight of the adrenal gland of Citellus tridecemlineatus.

Seasonal fluctuations in the weight of the adrenal gland show, in both sexes, a distinct seasonal trend. The curve, as plotted against the months of the year, gradually ascends from the low winter point to a May peak, and thence slowly returns to the winter bottom (chart 1).

Determinations of the cortex-to-medulla ratio were made in glands representative each of a different season of the

year. The following method was used: each gland selected for this purpose was sectioned serially and transversely at 10 μ, and an outline drawing made on paper, by means of a camera lucida ('Zeichenapparat nach Abbé'), of every tenth section of that gland. The drawing, in each case, included the circumference of the entire section as well as of its medullary portion whenever present. The total area of the magnified sections drawn for a given gland was then determined by means of a Keuffel-Esser planimeter, and similar measurements were taken of the corresponding medullary area. The number of planimeter units of area contained in the magnified medullary portion as it appeared in the drawings (designated in table 1 as 'M') was subtracted from the number of similar units obtained for the entire gland, and the remainder gave the number of planimeter units of cortical area (symbolized by 'C' in table 1) in the outline drawings of the adrenal under consideration. The ratio of the numbers of planimeter units obtained for these enlarged areas of cortex and medulla, respectively, being also the ratio of the true volume of cortex to the true volume of medulla, was simplified by reducing the latter to unity (these ratios, in their simplified form, appear in table 1 in the column headed 'C/M'). These determinations must not, of course, be regarded statistically: they merely offer suggestive comparisons. Thus, they indicate that, in both sexes, the increase in the weight of the gland in the spring is due chiefly to enlargement of cortical volume. It is noteworthy, moreover, that this increase in the volume of the cortex is greatest during the period of the highest sexual activity and, in the female, also during gestation (table 1).

The data given in table 1 reveal an interesting relationship. On the male side, for instance, the cortex-medulla ratio in the winter animal with abdominal testes and undeveloped accessories[4] (no. 741) was 5.9:1 while in the spring animal

[4] Throughout the present report, all references to the sexual condition of the males and females studied are based on diagnoses made by Mr. L. J. Wells and Mr. Geo. F. Simmons, respectively.

TABLE 1

Cortex-medulla ratios in the adrenal at various periods of the annual sexual cycle

ANIMAL (M-MALE, F-FEMALE)	DATE SACRIFICED	C	M	C/M	REMARKS
M-741	12/ 3/33	12,042	2067	5.9 : 1	Hibernating, sexually low
M-749	1/ 6/34	10,603	1866	5.7 : 1	Laboratory adult, sexually low
M-627	4/30/32	21,243	3110	6.8 : 1	Testes and accessories still high, beginning to regress
M-750	1/ 6/34	37,934	3415	11.1 : 1	Laboratory adult, near breeding condition
F-268	11/21/32	11,699	2263	5.2 : 1	Sexually low
F-121	4/29/32	20,532	3120	6.6 : 1	In full oestrum
F-600	5/31/33	21,033	1923	10.9 : 1	Pregnant, near term

no. 627, just past breeding condition, the corresponding ratio amounted to 6.8 : 1. Particularly instructive was the case of males no. 749 and no. 750. Both were laboratory confined adult males, i.e., males which had passed at least once through a complete annual sexual cycle; both were sacrificed on the same day, namely, on January 6, 1934. Whereas, however, the former had small abdominal testes and low accessories, the testicles of the latter were of near maximum or maximum size and confined within the densely pigmented scrotum, the epididymis contained quantities of motile spermatozoa, and the remaining accessories were well advanced in their upward development—a contrast commonly observed among laboratory confined adult males during January and invariably paralleled by a corresponding difference in the size of the adrenals. Thus, the adrenal of male no. 749 weighed only 9 mg. and gave a cortex-medulla ratio of 5.7 : 1, while the respective gland of male no. 750 weighed 22.5 mg., and its cortex-medulla ratio was 11.1 : 1.

On the female side, the ratio of cortex to medulla in the sexually low November animal no. 268 was 5.2 : 1; in the April animal, which was in full oestrum, it rose to 6.6 : 1; and in female no. 600, in late pregnancy, it jumped further to 10.9 : 1.

A critical investigation into the immediate causes of the increase in cortical volume during the period of high sexual development was not undertaken as it would involve a prohibitive amount of cell counting. In the spring, with the onset of breeding activity, certain marked histological changes occur, however, in the adrenal cortex, which can account, at least partly, for the increase in volume of the latter. The cortex, as a whole, becomes highly hyperaemic. The zona reticularis widens and becomes differentiated into two distinct sub-zones which, until the physiological significance of this differentiation is known, we shall designate by the non-committal terms of 'reticularis A' and 'reticularis B.' The latter corresponds to the inner sub-zone which, in arrangement and cell structure, constitutes the typical reticularis described above and present in the gland throughout the year. The outer sub-zone, the 'reticularis A,' is, as we shall see later, transitory and attains its highest development in both sexes during the peak of sexual development. It forms chiefly, and perhaps wholly, at the expense of the fasciculata and still retains the fasciculate arrangement of its cell cords. Nevertheless, cytologically, each individual cell of this sub-zone is a reticularis cell, its reticularis characters, though, being exaggerated, and the entire cell apparently reaching a high degree of specialization.

A well-developed 'reticularis A' cell (fig. 5) is very large, often measuring 60 μ in diameter. It is polyhedral, cuboidal, or roughly triangular in shape. Its nucleus is similar to that of a typical fasciculata cell, the general increase in the size of the cell being due to cytoplasmic expansion. Surrounding the nucleus and often separated from it by a narrow protoplasmic ring, is a large mass of exceedingly dense, deep staining pigment-like material often bearing upon it a crescentic adnuclear cap reticular in structure. This dense pigment-like mass fills nearly the entire cytoplasm, leaving, however, a clear peripheral ring whose nature and physiological significance were not disclosed. When first noted in sections prepared from Bouin-fixed material, this peripheral ring was

thought to be a fixation artifact. The same structure appeared, however, in glands fixed in formalin-Zenker or Regaud's fluid as well as in lipoid-demonstrating preparations. Consequently, the peripheral ring is not a fixation product or a result of lipoid removal. Aside from the tendency of Bouin fixation to emphasize the contrast in staining properties between the light perinuclear and peripheral rings, on one hand, and the dark pigment-like mass, on the other, there was a surprisingly close correspondence in the cytological appearance of the 'reticularis A' cell regardless of the fixing and staining methods employed. Moreover, the essential constituents of the cell in question were identifiable even in unstained sections. The inevitable conclusion, then, is that the configuration of the substances of various densities in the 'reticularis A' cell is due to pre-existing chemicophysical differences, and not to artificially produced local disturbances of the lipoid-protein equilibrium. In its low lipoid content, the 'reticularis A' cell resembles the typical reticularis ('reticularis B') cell.

The width of the 'reticularis A' sub-zone, in spring adrenals, varies from gland to gland. It usually ranges between three and fifteen cells in thickness and is inversely proportional to the width and degree of general development of the fasciculata. Whereas the adrenal gland of the female never seems to exhaust its fasciculate zone—which, incidentally, is better developed throughout the year than in the adrenal of the male—a high degree of development of 'reticularis A' in the adrenal of the male may be found in association with the virtual suppression of the typical fasciculate zone. In cases where both these zones are present, the transition between the zone containing typical fasciculata cells and that consisting of 'reticularis A' cells is gradual and expresses itself in a progressive loss of lipoids and acquisition of the pigment-like dark material characteristic of reticularis cells. Whether 'reticularis B' also contributes to the formation of 'reticularis A,' remains thus far unknown, though unlikely. The process—

Fasciculata → 'Reticularis A' → 'Reticularis B'

seems to be irreversible.

'Reticularis A' shows a most remarkable seasonal behavior. In the male, it reaches its highest development shortly following emergence from hibernation, and continues on a high level during May. It persists, but is decidedly on the decline during the summer, and normally disappears altogether about October. From October to January it is either absent or poorly developed in the adrenals of both immature and adult males. In January, its upgrade progress begins, and, following a gradual increase in width and accentuation of its specific characteristics, it returns to a new peak early in April, and the annual cycle is then repeated. This rhythm is even more pronounced in the female. During the late autumn and winter, females, both immature and adult, show no trace of 'reticularis A' in their adrenals (figs. 1 and 3). Females captured in the early spring, upon their emergence from the winter burrow, had adrenals with a well-developed outer reticularis ('reticularis A'), but this sub-zone was observed to attain its highest degree of development in May, i.e., during pregnancy. The 'reticularis A' of pregnancy is undoubtedly more conspicuous, both qualitatively and quantitatively, than at any other period of the annual cycle in untreated animals of either male or female sex (figs. 2 and 4). Following parturition, this transitory sub-zone undergoes a gradual regression until, in October, it becomes no longer distinguishable in cell characters from 'reticularis B.'

EXPERIMENTAL

a. Administration of gonad stimulating substances

All gonad stimulating substances used proved, on the whole, excellent stimulants for the adrenal cortex. In order of stimulating power with respect to the adrenal cortex, they were: pituitary hebin and suspension of whole sheep pituitary powder (of these unstandardized materials, probably large overdoses were administered), implants of anterior pituitary

lobes of rats, untreated human pregnancy urine (chiefly of late pregnancy), and antuitrin S. None of these substances seemed to affect histologically the adrenal medulla, at least not in a manner demonstrable by ordinary technique. No stimulation, either in the cortex or in the medulla, was obtained from implantation of guinea pig pituitaries, even when long time castrates were used as donors.

A significant increase in the weight of the adrenal takes place following the administration of the pituitary gonadal stimulants. In a series of eighteen experimental females, the adrenal gland from a treated animal weighed, on the average, 20.3 mg. against 9.7 mg. which constituted the mean adrenal weight in an equal number of comparable untreated animals. Although the number of males treated with anterior pituitary was insufficient for a satisfactory statistical study, yet also here indications point to an enormous increase in the weight of the adrenal gland as a result of the treatment.

A similar, statistically significant increase in adrenal weight followed the administration of raw human pregnancy urine and antuitrin S (tables 2 and 3, respectively).

Quantitative determinations of cortex-medulla ratios show that whereas the adrenal medulla may or may not increase in volume as a result of administration of the gonadal stimulants used in this investigation, the cortex enlarges tremendously (table 4). This quantitative effect seems to be largely, if not wholly, due to direct stimulation of the adrenal by the gonad stimulating substances in question, or at least not through the gonads, since the same high degree of increase in cortical volume was obtained by injection of sheep pituitary suspension into several spayed females.

Histologically, the increase in cortical volume under the influence of the gonadal stimulants administered, can be traced chiefly to a considerable, most evident increase in the number of cells of the adrenal cortex, particularly striking in the reticularis; to an increased vascularization of the gland as a whole; to cellular hypertrophy in the glomerular and fasciculate zones; and to the differentiation of a wide, beautiful

'reticularis A,' irrespective of season. Hypertrophied glomerulosa cells elongate in a direction perpendicular to the adjacent trabecula, and their cytoplasm becomes intensely vacuolated due to an increase in lipoid content. Each cell of the fasciculate zone also expands in all directions, and its lipoid load is likewise increased. In the case of several males injected with pregnancy urine, however, the fasciculate zone was completely eliminated by the over-developed 'reticularis

TABLE 2

Effect of injection of untreated human pregnancy urine on adrenal weight

EXPERIMENTAL ANIMALS						UNTREATED CONTROLS			
Animal	Amount of daily injection	Injected for (days)	Sacrificed date	Body weight	Weight of adrenal	Animal	Sacrificed date	Body weight	Weight of adrenal
	cc.			*gm.*	*mg.*			*gm.*	*mg.*
♂ 35	2	40	12/12/31	167	10.0	♂ 43	12/12/31	196	7.4
♂ 36	2	33	12/ 5/31	118	14.4	♂ 744	12/ 3/33	226	7.6
♂ 322	2	22	8/26/32	148	14.2	♂ 330	8/22/32	180	16.8
♂ 323	2	30	9/ 3/32	120	34.7	♂ 721	9/ 9/33	234	8.6
♂ 325	1	15	8/24/32	96	17.9	♂ 717	8/18/33	154	12.3
♂ 326	1	31	9/10/32	122	22.2	♂ 727	9/ 9/33	150	7.5
♂ 331	2	10	9/15/32	114	11.4	♂ 728	9/ 9/33	152	8.2
♂ 332	2	13	9/18/32	116	21.1	♂ 715	8/18/33	144	10.2
♂ 334	2	12	9/18/32	122	25.3	♂ 716	8/18/33	134	10.1
♂ 335	2	22	9/28/33	154	13.4	♂ 386	10/30/32	142	6.9
♂ 336	2	47	10/29/32	150	20.8	♂ 387	10/30/32	168	9.2
♂ 354	2	41	11/19/32	144	15.6	♂ 734	11/19/33	202	11.0
♂ 359	2	41	11/19/32	192	17.3	♂ 735	11/19/33	230	9.5
♂ 360	2	41	11/19/32	148	9.1	♂ 736	11/19/33	228	9.2
♂ 362	2	41	11/19/32	156	11.3	♂ 424	10/17/32	206	9.0
♂ 364	2	38	11/19/32	196	8.4	♂ 737	11/19/33	216	10.2
♂ 396	2	27	12/10/32	152	13.9	♂ 435	12/28/32	195	6.7
♂ 397	2	27	12/10/32	173	16.4	♂ 742	12/ 3/32	272	11.5
				Mean:	16.5[1]			Mean:	9.6[1]
♀ 12	2	26½	11/ 3/31	144	16.7	♀ 45	11/21/31	187	4.0
♀ 29	3	19	11/18/31	146	8.3	♀ 46	11/22/31	166	6.0
♀ 49	3	10	1/16/32	172	22.8	♀ 269	11/21/32	185	7.1
♀ 220	2	3	8/31/32	120	17.4	♀ 665	8/18/33	122	9.5
♀ 267	2	60	11/21/32	130	13.3	♀ 270	11/22/31	202	4.5
				Mean:	15.7[2]			Mean:	6.2[2]

[1] Difference of means is 4.2 times its standard error; statistical significance of increase is less than 0.0001.

[2] Difference of means is 3.67 times its standard error; statistical significance of increase is 0.0002.

A,' and even the glomerulosa began to show a distinct tendency toward deglomerulization, acquisition of the deeply staining material typical of reticularis cells, and general transformation in the 'reticularis A' direction (fig. 6). In many greatly expanded 'reticularis A' cells of such adrenals the nuclear chromatin becomes clumped and shriveled, and is retracted from the nuclear membrane.

It should be noted that pregnancy urine had a generally toxic effect upon the animals, the greatest majority of which lost considerable weight and became highly emaciated as a result of this treatment. The injection sites turned into foci of spreading infection. Four males even succumbed to the treatment. It is doubtful, however, whether the toxicity of urine was significantly involved in the adrenal changes observed, since antuitrin S, a non-toxic extract made from urine of pregnant women, gave results practically identical with those obtained by injection of pregnancy urine.

TABLE 3

Effect of injection of antuitrin S on the weight of the adrenal gland

EXPERIMENTAL ANIMALS						UNTREATED CONTROLS			
Animal	Amount of daily injection	Injected for (days)	Sacrificed date	Body weight	Weight of adrenal	Animal	Sacrificed date	Body weight	Weight of adrenal
	rat units			*gm.*	*mg.*			*gm.*	*mg.*
♂ 350	50–100	29	11/ 6/32	146	15.4	♂ 393	11/12/32	182	7.4
♂ 358	50–100	21	10/28/32	143	16.0	♂ 386	10/30/32	142	6.9
♂ 394	100	27	12/10/32	214	11.1	♂ 43	12/12/31	196	7.4
♂ 395	100	27	12/10/32	236	11.4	♂ 435	12/28/32	195	6.7
♂ 428	15	26	1/ 4/33	203	10.2	♂ 458	1/28/32	250	10.4
♂ 429	35	26	1/ 4/33	171	5.7	♂ 749	1/ 6/34	150	9.0
♂ 430	5	26	1/ 4/33	160	13.4	♂ 56	1/16/32	154	7.7
				Mean:	11.9[1]			Mean:	7.9[1]
♀ 277	5	7	12/12/32	136	10.6	♀ 59	1/29/32	204	8.1
♀ 279	25	7	12/12/32	168	15.9	♀ 301	12/31/32	113	9.6
♀ 280	50	7	12/12/32	166	13.5	♀ 57	1/27/32	193	4.3
♀ 282	10	7	12/12/32	132	15.0	♀ 303	1/ 1/33	147	5.0
♀ 288	1	7	12/12/32	143	11.5	♀ 268	11/21/32	150	6.0
				Mean:	13.3[2]			Mean:	6.6[2]

[1] Difference of means is 2.84 times its standard error; statistical significance of increase is 0.0058.

[2] Difference of means is 4.75 times its standard error; statistical significance of increase is less than 0.0001.

b. Administration of sex hormones

The subcutaneous injection, into males or females, of either testicular or ovarian (follicular) hormone resulted in a slight, statistically insignificant increase in the mean weight of the adrenal gland (tables 4 and 5), and in variable cortex-medulla ratios (table 6).

In males injected with testis hormone there is, generally, no increase in the width of the adrenal cortex and there is no evidence of stimulated cell proliferation. A well-differentiated 'reticularis A' is developed, however, regardless of season. Furthermore, the degree of stimulation of 'reticu-

TABLE 4

Effect of injection of testis hormone on the weight of the adrenal gland

EXPERIMENTAL ANIMALS						UNTREATED CONTROLS			
Animal	Amount of daily injection	Injected for (days)	Sacrificed date	Body weight	Weight of adrenal	Animal	Sacrificed date	Body weight	Weight of adrenal
	bird units			*gm.*	*mg.*			*gm.*	*mg.*
♂ 34	12.5	25	12/27/31	180	9.6	♂ 734	11/19/33	202	11.0
♂ 103	6.6	9	7/19/32	144	25.8	♂ 707	7/17/33	100	5.6
♂ 105	13.3	20	8/ 1/32	204	16.3	♂ 718	8/18/33	236	13.9
♂ 106	13.3	20	8/ 1/32	194	9.4	♂ 330	8/21/32	180	16.8
♂ 107	6.6	7	7/20/32	170	14.9	♂ 708	7/17/33	210	15.2
♂ 113	7.0	22	8/16/32	106	7.0	♂ 714	8/18/33	132	11.0
♂ 115	6.6	21	8/11/32	228	23.8	♂ 718	8/18/33	236	11.9
♂ 116	6.6	21	8/11/32	220	10.1	♂ 719	8/18/33	212	10.9
♂ 351	12.0	12	10/20/32	116	10.0	♂ 386	10/30/32	142	6.9
♂ 352	6.0	29	11/ 6/32	170	6.5	♂ 735	11/19/33	230	9.5
♂ 388	12.0	26	11/26/32	222	16.9	♂ 737	11/19/33	216	10.2
♂ 489	15.0	21	4/23/32	173	19.4	♂ 619	4/21/33	194	12.8
♂ 490	15.0	26	4/29/32	165	12.0	♂ 625	4/29/33	159	12.3
				Mean:	14.0[1]			Mean:	11.4[1]
♀ 27	10.0	19	11/18/31	176	7.0	♀ 45	11/21/31	187	4.0
♀ 28	20.0	19	11/18/31	154	7.8	♀ 46	11/22/31	166	6.0
♀ 291[2]	5.0	7	12/12/32	143	7.0	♀ 301	12/31/32	113	9.6
♀ 292[2]	15.0	7	12/12/32	126	6.8	♀ 303	1/ 1/33	147	5.0
				Mean:	7.2[3]			Mean:	6.2[3]

[1] Difference of means is only 1.35 times its standard error; increase not statistically significant.

[2] Bilaterally spayed; injections begun immediately following ovariotomy.

[3] Difference of means is less than its standard error; increase not statistically significant.

laris A' corresponds roughly to the amount of testis hormone administered. No noteworthy change in the lipoid content of the glomerular and fasciculate zones was observed.

The adrenals of males which had received injections of oestrin showed, peculiarly enough, effects similar to those exhibited by the corresponding glands of males injected with testis hormone. In all males of this series, with the exception of one doubtful case, the adrenal presented a well-developed 'reticularis A.' The correlation between the amounts of hormone injected and the degree of stimulation of the 'reticularis A' was, however, not so apparent as in the case of the testis hormone treatment. Moreover, the highest level of 'reticu-

TABLE 5

Effect of injection of oestrin on the weight of the adrenal gland

EXPERIMENTAL ANIMALS						UNTREATED CONTROLS			
Animal	Amount of daily injection	Injected for (days)	Sacrificed date	Body weight	Weight of adrenal	Animal	Sacrificed date	Body weight	Weight of adrenal
	rat units			*gm.*	*mg.*			*gm.*	*mg.*
♂ 32	12.5	32	12/ 5/31	161	11.8	♂ 43	12/12/31	196	7.4
♂ 33	25.0	25	12/12/31	150	10.0	♂ 744	12/ 3/33	226	7.6
♂ 84	25.0	34	5/14/32	178	15.8	♂ 88	5/14/32	140	18.3
♂ 84	12.5	34	5/14/32	160	16.9	♂ 89	5/14/32	162	13.5
♂ 112	25.0	17	8/11/32	96	8.5	♂ 714	8/18/33	132	11.0
♂ 327	25.0	11	8/23/32	116	27.7	♂ 715	8/18/33	144	10.2
♂ 328	25.0	7	8/19/32	74	12.6	♂ 716	8/18/33	134	10.1
♂ 345	25.0	29	11/ 6/32	196	13.1	♂ 734	11/19/33	202	11.0
♂ 346	12.5	29	11/ 6/32	214	15.4	♂ 735	11/19/33	230	9.5
♂ 347	12.5	29	11/ 6/32	91	13.1	♂ 736	11/19/33	228	9.2
♂ 349	25.0	29	11/ 6/32	181	13.2	♂ 803	10/21/33	228	11.2
♂ 487	25.0	26	4/29/33	128	14.9	♂ 625	4/29/33	159	12.3
♂ 488	25.0	26	4/29/33	136	17.3	♂ 624	4/29/33	200	17.7
				Mean:	14.6[1]			Mean:	11.5[1]
♀ 25	12.5	19	11/18/31	144	7.6	♀ 45	11/21/31	187	4.0
♀ 26	25.0	19	11/18/31	178	10.7	♀ 46	11/21/31	166	6.0
♀ 293[2]	1.0	7	12/12/32	136	7.6	♀ 301	12/31/32	113	9.6
♀ 294[2]	6.0	7	12/12/32	144	7.5	♀ 303	1/ 1/33	147	5.0
				Mean:	8.4[3]			Mean:	6.2[3]

[1] Difference of means is only 1.94 times its standard error; increase not statistically significant.

[2] Bilaterally spayed; injections begun immediately following ovariotomy.

[3] Difference of means is only 1.52 times its standard error; increase not statistically significant.

laris A' development was found, with one single exception, in animals captured and sacrificed during the spring and summer, i.e., at periods when a good outer reticular sub-zone is normally present in the adrenal. Obviously, the administration of oestrin in these cases may not have had any effect at all. It seems, therefore, that a more extensive series of oestrin injected males is necessary in order to establish the effect of follicular hormone on the adrenal cortex of the male Citellus.

One interesting fact, however, was revealed by the study of the adrenal in males injected with oestrin: the treatment caused injury, in some cases severe, to the cortical tissue. This injury was evidenced by the occurrence of large hemorrhagic areas in the peripheral zones, and by the prevalence of large intracellular vacuoles, of 'signet ring' cells and 'giant cells,' and of more or less extensive fibroses in the reticularis. The lipoid content of the glomerulosa and of the fasciculata was markedly increased.

Among the females, only four were injected with oestrin, and four with testis hormone. Four of these females (two injected with testis hormone and two with oestrin) were sacrificed in November, and the remaining four in December. Whereas in controls of this time of the year 'reticularis A' is invariably absent from the adrenal cortex, all four females injected with oestrin showed, in their adrenals, a well-developed outer reticular sub-zone. Of the four females injected with testis hormone, three had a fairly conspicuous 'reticularis A' in their adrenals, while one had none. Whether the effect, in the three animals which showed positive results, was due to the male hormone contained in the testicular extract or to the oestrogenic fraction whose presence was likewise demonstrated in the particular preparation used, is not known.

No effect of sex hormone administration, in either male or female, on the adrenal medulla could be demonstrated.

c. Castration

The effects of castration of some duration (of at least 1 month) on the adrenal cortex were identical in both sexes. They expressed themselves chiefly in the manifestation of intensive cell destruction in the reticularis, as well as in the complete or nearly complete suppression of 'reticularis A.' The most typical 'castration picture' in the adrenal cortex consisted of: a wholesale destruction of the innermost rows

TABLE 6

Cortex-medulla ratios in the adrenals of experimental animals

ANIMAL	SACRIFICED DATE	TREATMENT	C	M	C/M
♂ 741	12/ 3/33	Untreated December control	12,042	2067	5.9: 1
♂ 54	1/ 5/32	Pituitary hebin, 1½ cc. daily, 19 days	37,486	2808	13.3: 1
♂ 35	12/12/31	Pregnancy urine, 2 cc. daily, 40 days	26,466	2626	10.1: 1
♂ 358	10/28/32	Antuitrin S, 50 r.u. daily, 19 days + 100 r.u. daily, 2 days	25,375	2858	8.9: 1
♂ 21	10/31/31	Testis hormone, 12½ bird units daily, 20 days	13,856	2511	5.5: 1
♀ 268	11/21/32	Untreated November control	11,699	2263	5.2: 1
♀ 47	1/ 5/32	Pituitary hebin, 1½ cc. daily, 20 days	53,891	2873	18.8: 1
♀ 194	8/ 1/32	Sheep pituitary suspension, 1 cc. daily, 11 days	22,150	1765	12.5: 1
♀ 192	8/ 1/32	Ovariectomy + sheep pituitary suspension, 1 cc. daily, 11 days	26,880	2194	12.3: 1
♀ 49	1/16/32	Pregnancy urine, 3 cc. daily, 10 days	43,953	3005	14.6: 1
♀ 280	12/12/32	Antuitrin S, 50 rat units daily, 7 days	21,956	1894	11.6: 1
♀ 25	11/18/31	Oestrin, 12½ rat units daily, 19 days	19,236	1795	10.7: 1

of reticularis cells and their replacement by a fibrous ring surrounding the medulla; degeneration foci distributed throughout th reticular layer in the form of 'green cells' ('giant cells'); a total absence of 'reticularis A' at all seasons of the year. The 'giant cells' are designated by the writer as 'green cells' on the basis of the numerous fine, highly refractile granules with which these cells are packed and which stain a brilliant green with Mallory. These granules are probably composed of pigment whose intense yellow blends with the anilin blue of the Mallory stain to produce their

characteristic green hue. The 'green cells' become—in size, number, and mode of staining—a sharply outstanding element in the reticularis of castrates (fig. 7). Whereas, in the adrenals of untreated animals, these cells are rarely present and never conspicuous, in the corresponding glands of castrates they often comprise each an area normally occupied by at least half a dozen cells, and their number may exceed ten per section 4 μ in thickness. Each of these formations often contains remnants of as many as three nuclei, but may also be seen—apparently at a more advanced stage—lacking any nuclear remains whatever.

No consistent change in the quantity of lipoids in the adrenal cortex was observed following castration. Similarly, no response was evident in the medulla.

DISCUSSION

The present investigation comprises seasonal observations and experimental studies involving some 350 males and over 300 females. Its results enable the writer to add several suggestive facts to the voluminous and constantly growing body of data regarding a definite functional relationship between the adrenal cortex, on one hand, and the gonads, on the other. For lack of space, the immense literature on the subject will not be reviewed here. Suffice to say that evidence has been forthcoming from three chief sources: clinical facts, experimental results, and normal histology and physiology. The clinical evidence is excellently reviewed by Glynn ('12 and '21) and summarized in a later publication by Goldzieher ('29). It consists chiefly of the genital syndrome accompanying hypernephromata (although the relationship between hypernephromata and the adrenal cortex has not been, as yet, critically demonstrated) as well as of some regressive changes in the reproductive system associated with adreno-cortical atrophy or hypoplasia. Some experimental evidence is reviewed in the introduction to the present report. This evidence is still contradictory, but, with improvement in technique, we shall, no doubt, obtain a greater agreement also in

this field of research. As for observations on the normal morphology of the adrenal cortex in relation to sexual phenomena, several important investigations, notably those of Kolmer ('12) on the guinea pig and Tamura ('26), Masui and Tamura ('26), Deanesly ('28), and Howard-Miller ('27) on the mouse, suggest a definite correlation between the adrenal cortex and the gonads. Just how essential this relationship is, we are still unable to say; in a more recent publication by Howard ('30), she is inclined to minimize the significance of the interaction in question. The fact, however, that such relationship does exist and that the main changes in the adrenal cortex associated with various phases of sexual activity are localized in the reticular zone, is in complete accord with the results of the present work. The study of the adrenal cycle in Citellus tridecemlineatus also offers definite proof against the view held by some of the older histologists and apparently still maintained by Tamura ('26) that every zone of the adrenal cortex has an independent origin as well as an independent course of development. This investigation reveals, on the contrary, that in the matter of zonal differentiation and zonal equilibrium in the cortex of the adrenal gland we deal with a highly dynamic situation controlled and regulated largely, if not wholly, by factors endocrine in nature.

SUMMARY AND CONCLUSIONS

A definite interrelationship exists, in the thirteen-lined ground squirrel, between the adrenal cortex and the reproductive cycle. Thus, a significant increase in adrenal size and weight traceable to cortical hypertrophy takes place in the adrenals of both male and female ground squirrels during the breeding period or as a result of artificial stimulation of the gonads and reproductive accessories at the season of sexual inactivity. Histologically, this cortical hypertrophy is associated mainly with the expansion of the reticular zone and the differentiation, within it, of a characteristic, highly developed outer sub-zone termed 'reticularis A' and described

fully. Castration of at least 1 month's duration leads, in the adrenal of either sex, to degenerative changes in the zona reticularis.

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PLATES

PLATE 1

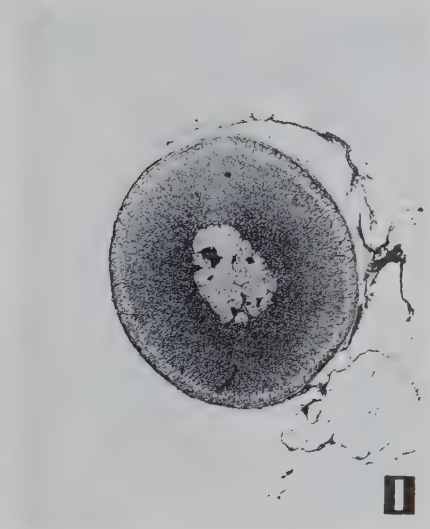
EXPLANATION OF FIGURES

1 A central section through the adrenal of a normal, untreated female sacrificed in December. A typical winter gland. Bouin; Krichesky-Mallory. × 28¾.

2 A central section through the adrenal of a female in mid-pregnancy, sacrificed in May. A typical spring gland. Note the middle cortical layer of large cells with dark central portions and light peripheries ('reticularis A'). Bouin; Krichesky-Mallory. Same magnification as in figure 1 (× 28¾). Compare the size of both sections.

3 Portion of the section represented in figure 1 under higher magnification, showing the entire thickness of the cortex. Note the wide fasciculate zone and the total absence of 'reticularis A.' × 170.

4 Portion of the section represented in figure 2 under higher magnification, showing the entire thickness of the cortex. Note the wide, conspicuous 'reticularis A.' × 105.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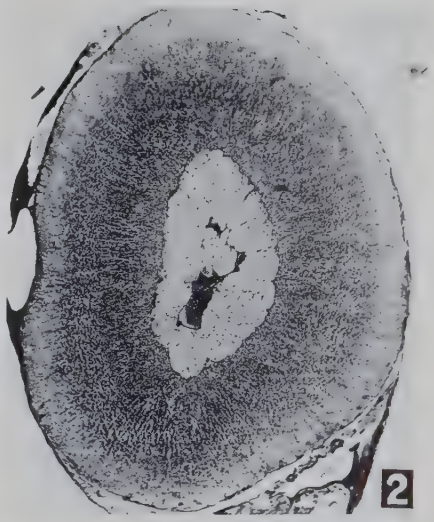
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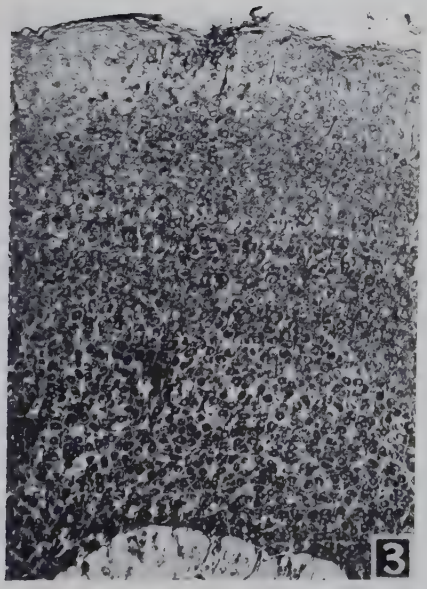
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PLATE 2

EXPLANATION OF FIGURES

5 Portion of 'reticularis A' from the section represented in figures 2 and 4, greatly magnified. Note the dense pigment-like material surrounding the nucleus, and the perinuclear and peripheral rings. × 1025.

6 Portion of a section through the adrenal of male no. 35 treated with pregnancy urine (two daily injections of 1 cc. each for 20 days). Note the elimination of the fasciculate zone by the enormously expanded 'reticularis A' and the beginning transformation of the glomerulosa in the 'reticularis A' direction. Bouin; Krichesky-Mallory. × 285.

7 A greatly magnified portion of the 'reticularis B' near the medullary border (which appears at lower left) showing cell degeneration following bilateral castration. Female no. 205, spayed for 2½ months. Note the 'green cells' (giant cells) with pycnotic nuclei. Bouin; Krichesky-Mallory. × 1025.

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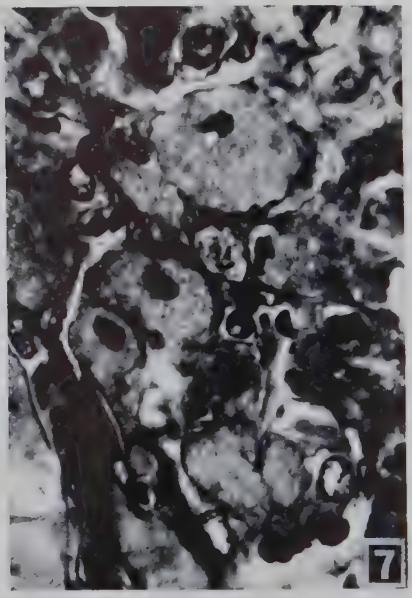
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FURTHER STUDIES ON THE INFLUENCE OF SUCKLING

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SEVEN FIGURES

It has recently been shown, in experiments on rats and mice (Selye, '34), that cessation of secretion and involution of the mammary glands which follow removal of the litter are due, not to the accumulation of milk within the gland, but rather to the withdrawal of the stimulus of suckling; and it has also been shown that the application of this stimulus to normal adult females leads to development of the mammary glands and eventually to milk secretion (Selye and McKeown, '34 a), and to prolonged, almost continuous dioestrus, with changes in the uterus, ovaries, and hypophysis (Selye and McKeown, '34 b) which led us to describe the condition as 'suckling pseudopregnancy.' It seemed desirable to extend these studies, and to reconsider certain current ideas of the physiology of lactation, in the light of the emphasis now placed on the influence of the act of suckling. It is well known, for instance, that lactation cannot be maintained indefinitely, even though the milk is continually removed from the mammary gland. Thus in dairy cattle, even if the animals are regularly milked, the milk production eventually decreases, and involutionary changes in the gland have been made in other species.

In a group of eight lactating mice, we maintained the stimulus of suckling for a considerable period beyond the usual span by replacing the growing litters from time to time by very young ones; in this way it was possible to be

sure that a strong nursing stimulus was continually present. It was observed that, after about 8 weeks, such animals were unable to nourish their young, and from this time onward the litters were exchanged daily with the actively nursing litters of normally lactating mothers, so that death from inanition was averted. Even after a very long period (in two cases as long as 68 days) normal oestrous cycles had not reap-

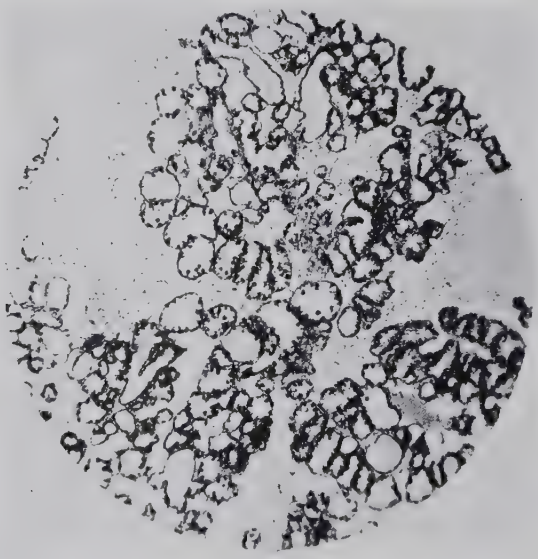

Fig. 1 Low magnification of the mammary gland of a mouse taken during the fourth week of lactation. Excrescences of epithelial cells invade the lumen of the acini.

peared. The vaginal smears indicated that the first recurrence of oestrus occurred in the third week after parturition, that is at the time at which it has usually been reported in the normal course of lactation. This oestrous period was followed, however, not by normal cycles, but by successive periods of dioestrus each of from 12 to 17 days' duration, separated the one from the other by a single appearance of oestrus. In a similar experiment, Crew and Mirskaia

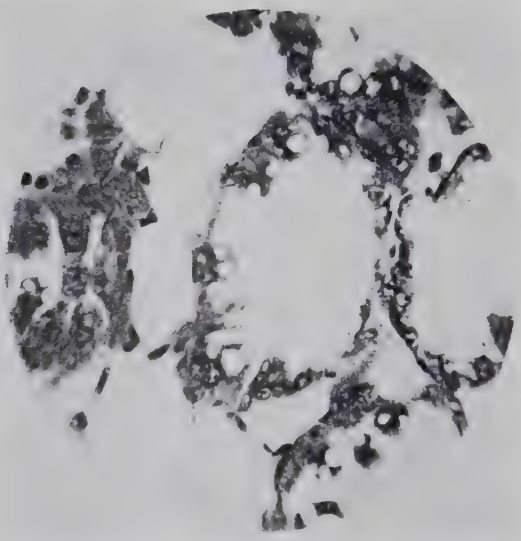

Fig. 2 High magnification of an acinus in the mammary gland shown in figure 1. Several cells are missing from the epithelial lining.

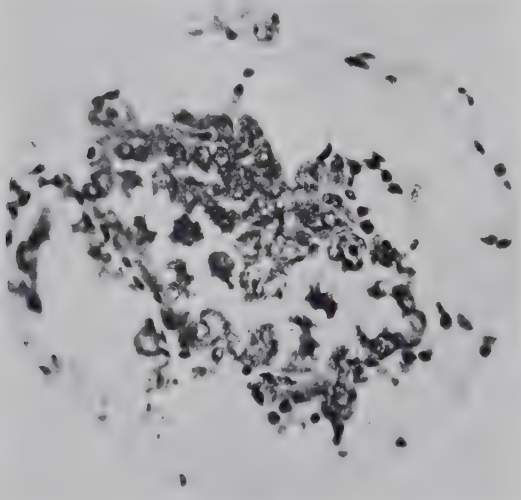

Fig. 3 High magnification of another acinus in the mammary gland shown in figure 1. Numerous cells lie free in the lumen.

('30) failed to observe this phenomenon, since they abandoned their studies at the first reappearance of the cornified vaginal smear; and Parkes ('26) allowed his animals to copulate and become pseudo-pregnant at each recurrence of oestrus.

In these same animals, we followed the changes in the mammary gland by taking biopsy specimens for histological

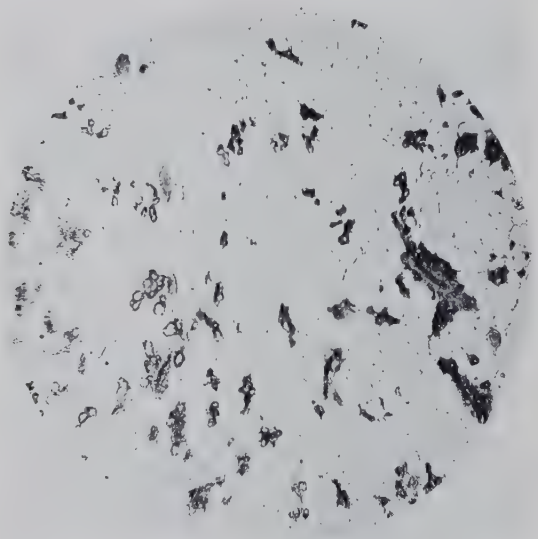

Fig. 4 Low magnification of the mammary gland of a mouse on the forty-second day of lactation. The parenchyma has undergone complete involution and only the duct system is left.

study. Degeneration was quite marked in some cases by the end of the fourth week, and in all at least the beginning of retrogression could be seen at this time. Figure 1 illustrates the histological picture of this type of involution, which is quite characteristic and distinct from the picture of physiological involution after weaning. The first sign of degeneration is the budding-out of cells or cell-complexes into the lumina of the acini; this is often so marked that the gland assumes a papillomatous appearance. At this stage all

the epithelial cells of the acini become very markedly basophilic, so that the nucleus is hardly distinguishable from the cytoplasm. At a later stage, the epithelial cell buds are shed and lie free in the lumen (figs. 2 and 3) some weeks later still, the whole parenchyma of the gland disappears, and only the duct system remains (fig. 4). We are under the impression that the budding of cells into the lumen is

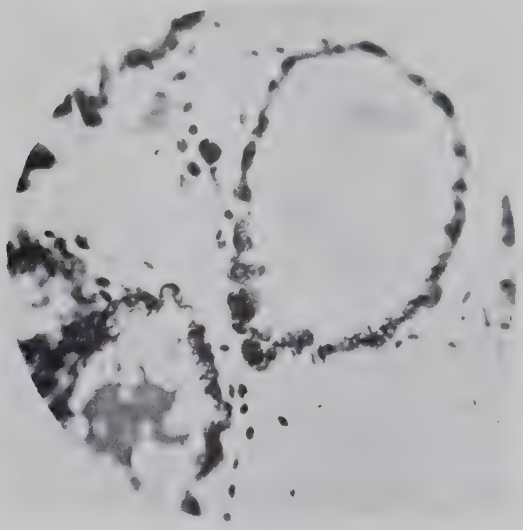

Fig. 5 High magnification of an acinus during the fourth week of lactation. Some of the epithelial cells extrude toward the stroma.

sometimes accompanied by a budding toward the stroma, that is in the opposite direction; for in degenerating glands we have frequently observed cells extruding from the epithelial lining into the interacinar spaces (fig. 5), and cells and cell-complexes of epithelial type and possessing basophilic cytoplasm may often be found in the stroma (fig. 6). Vacuoles eventually appear in these free epithelial cells, which come to assume the appearance of fat cells (fig. 7).

It is evident from this experiment that although the stimulus of suckling, if vigorously applied, can maintain a condition of almost continuous dioestrus for long periods in normally lactating mice, it cannot keep the mammary gland in a state of full development and function indefinitely.

We have described elsewhere (Selye and McKeown, '34 a and b) 'suckling pseudo-pregnancy,' induced by applying a vigorous nursing stimulus to normally cyclic adult female

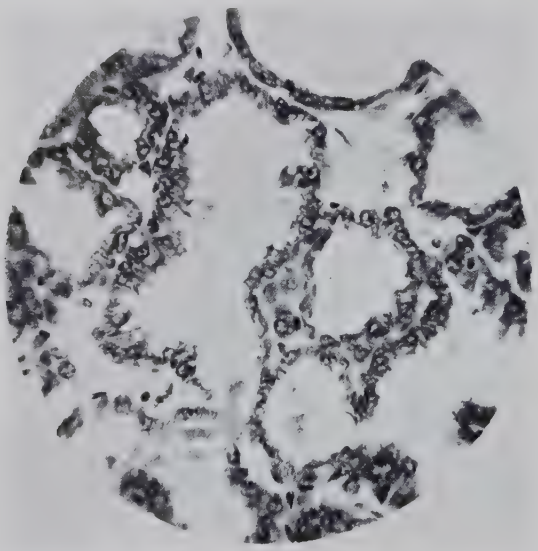

Fig. 6 Another section of the mammary gland shown in figure 5. In the center of the field several cells of epithelial type similar to the cells lining the wall of the acini are seen in the stroma.

rats and mice. In a recent repetition of these studies, on a group of six normally cyclic mice, we noted carefully when the act of suckling actually began. Biopsy specimens of the mammary glands taken at intervals thereafter showed that duct development had just begun on the third day; the gland was quite well developed on the fourth day in half the animals, and on the eighth day in all. The mice were sacrificed on the twelfth day, by which time one out of the six had actually commenced milk secretion.

We have pointed out (Selye and McKeown, '34 b) that 'suckling pseudo-pregnancy' differs from the 'copulation pseudo-pregnancy' produced by sterile coitus in that the former does not follow inevitably from one single stimulus, but requires that the nursing stimulus be constantly maintained. A further point of distinction deserves mention at this time: we agree with previous workers that sterile copulation is usually followed by proliferation of the mammary

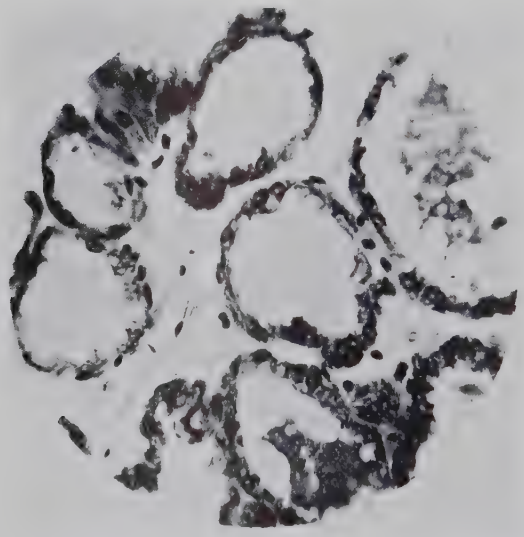

Fig. 7 Vacuolized cells in the stroma of the mammary gland shown in figures 5 and 6. Numerous intermediate stages between the typical epithelial cell and this vacuolized cell may be found in the stroma at this stage.

gland, in the rat; but while those who have studied this phenomenon in the mouse (Turner and Gomez, '33; Cole, '33) apparently regard it as regularly present, we have been unable to convince ourselves that this is the case. In a series of ten cyclic female mice which were mated with vasectomized males, and which all showed the usual prolongation of dioestrus, biopsy specimens of the mammary glands showed evidence of proliferation in only two instances. It

was found, however, that if the females were left with the sterile males for several weeks, mammary development did occur more frequently. We have never failed to observe mammary development in suckling pseudo-pregnancy in rats or mice.

Hypophysectomy performed during normal lactation leads promptly to cessation of secretion and involution of the glands both in rats (Collip, Selye and Thomson, '33) and in mice (Selye, Collip and Thomson, '33). We therefore hypophysectomized a rat and a mouse on the sixth day of suckling pseudopregnancy, when histological examination of samples from the mammary glands showed that considerable development had taken place; further samples, taken 5 days later, showed complete involution, although the suckling stimulus had been actively maintained in both animals; the corpora lutea also showed retrogressive change.

It is well known that ovariectomy, on the other hand, has no influence upon normal established lactation. We were therefore interested to know whether the ovary was necessary for the changes which we had observed to be brought about under the influence of the suckling stimulus in the normal animal. Four adult, normally cyclic mice were ovariectomized and supplied with actively nursing litters, which we maintained by exchanging them daily for the young of a normally lactating mother. The animals were sacrificed after 13 days, and the sections showed that the mammary glands and uterus were not affected by the suckling stimulus, but were identical with the organs of three castrate controls which had not been given litters.

It has previously been shown (Selye, '34) that if, in lactating animals, the escape of milk is prevented by transcision of all galactophores, the continued stimulus of suckling prevents involution of the mammary glands from occurring as rapidly as it does when the nipples are destroyed or the young removed. This delay in involution does not depend upon the ovaries; for in five normally lactating mice in which we performed bilateral ovariectomy as well as transcision of

all milk ducts, we found by examination of mammary gland specimens from time to time that involution occurred more slowly than in four controls which were weaned and ovariectomized on the same day of lactation. From this and the preceding experiment, we conclude that the nervous stimulus of nursing probably exerts its trophic effect on the lactating mammary gland by way of the pituitary. Additional evidence for this theory has been discussed elsewhere (Selye, Collip and Thomson, '34).

Although trancision of the galactophores does not lead to rapid involution if the suckling stimulus is maintained, nevertheless, the pressure of the accumulating milk eventually leads to degenerative changes within the mammary gland (Selye, '34). This observation was confirmed on a fresh series of six lactating mice, in which the operation was performed the day after parturition. When the pressure of milk had at least produced atrophy (much later than in the weaned controls), the glands were unresponsive to the stimulus of suckling. At a still later date, however, it was observed that the responsiveness of the glands to the stimulus of suckling was to some extent restored, although the degree of development attained was markedly inferior to that seen in normal mice when the stimulus of suckling is first applied.

SUMMARY

1. If the stimulus of suckling is maintained by frequently supplying fresh litters to normal lactating mice, active lactation may continue for about 2 months, after which an unusual type of involution of the mammary gland occurs. In spite of this involution, normal oestrous cycles do not reappear, the animals showing successive long periods of dioestrus, each lasting from 12 to 17 days.

2. The application of the suckling stimulus to normally cyclic mice leads to inhibition of oestrus to development of the mammary glands. This response is unobtainable in mice previously ovariectomized, and cannot be maintained after hypophysectomy.

3. Copulation pseudopregnancy, unlike suckling psedopregnancy, is not always attended by mammary development in the mouse.

4. The stimulus of suckling maintains secretory activity in the mammary gland for some time after escape of milk is prevented by transcision of all galactophores; this maintained activity may be seen even in the absence of the ovaries.

5. The pressure of the accumulating milk after transcision of galactophores eventually causes atrophy of the glands, which then become unresponsive to the stimulus of suckling; later, however, they regain in part the capacity to respond to this stimulus.

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THE PRENATAL INCIDENCE, STRUCTURE AND DEVELOPMENT OF SOME HUMAN SYNOVIAL BURSAE

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EIGHT FIGURES

In this investigation, the subacromial, the prepatellar, and the subcutaneous olecranon bursae were studied in a series of human fetuses by means of gross dissection and also in microscopical preparations. The prepatellar and olecranon subcutaneous bursae were selected because they were probably as constant as any of the subcutaneous bursae and are quite typical. The subacromial was selected as a representative deep bursa. The subfacial and the subtendinous prepatellar bursae were observed along with the subcutaneous.

The fetuses were selected on the basis of size and preparation, the latter being the more important criterion. In the first series, 200 fetuses were used, ninety females and 110 males. They varied in age from 3 months to full term, and, with the exception of those in the third month, there were at least seventeen fetuses from each month. For the most part the condition of this material was very good. The smaller fetuses had been preserved in 10 per cent formalin, and the preservation was uniformly excellent. The larger fetuses had been embalmed through the umbilical vein and then kept under oil or water, and the condition of these was in some cases not so good as that of the younger ones.

A cavity was regarded as a bursa if it had an inner surface which differed from the surrounding connective tissue. Injections were not made for fear that the fluid would enlarge

a pre-existing space or form one where none existed before. Microscopic sections were not made of this series, but the shoulders of twenty well-preserved fetuses ranging in length from 20 to 26 mm. were examined microscopically. The previously fixed formalin material was embedded, using the celloidin-paraffin method of Bartelmez, as given to me by Professor Markee, and sectioned frontally. One section in every 100 μ was mounted and stained with either hematoxylin and eosin or with hematoxylin and a mixture of orange G and anilin blue in a weak phospho-molybdic acid solution (Mallory II).

The soft tissues over the olecranon and patella in a small series of older fetuses, ranging in age from the fourth month to full term, were similarly examined to guard against the possibility that the bursae in these regions had been overlooked in the dissection.

In all cases, a crown-rump (CR) measurement was taken of the fetuses before any dissection was done and these measurements will be given subsequently in preference to menstrual age, because of the uncertainty still existing concerning the relationship of age and length in the early fetal and particularly in the late embryonic periods. When it is necessary to refer to fetus or embryo in my discussion, I shall follow Streeter's conclusion that any specimen with a CR length of less than 23 mm. is an embryo.

In the B.N.A. both a bursa subacromialis and a bursa subdeltoidea are recognized. While either one or both of these bursae may exist in the same adult shoulder, this condition is much rarer than the occurrence of one bursa which lies under both the acromia process and the deltoid muscle. In the literature and textbooks this bursa is loosely named 'subdeltoid' or 'subacromial'; even though there may be a subacromial bursa proximally or a subdeltoid bursa distally, in the same shoulder.

Since I only saw a bursa arise under the tip of the acromion, I shall use the term subacromial, but without the implication that two bursae never occur in this region. It is well known

that both a subacromial and a subdeltoid bursa may exist in the same shoulder in the adult, and I have also observed them rarely in the same shoulder of a fetus.

In the fifth edition of Cunningham's Anatomy, 1923, Paterson wrote, "The subacromial bursa intervenes between the middle fibers of the deltoid, the acromion, and the coracoacromial ligament, on the one hand, and the supraspinatus, infraspinatus, and subscapularis tendons on the other," and Hepburn added that, "It greatly facilitates the movements of the proximal end of the humerus against the inferior surface of the coraco-acromial arch."

In fetuses older than the fourth month, the bursa is somewhat rectangular in shape; since it usually lacks a prolongation over the lesser tuberosity, and does not extend far enough dorsally to overlie the tendon of the infraspinatus. The proximal extremity which is rather ill-defined, is encountered at varying distances under the acromion, depending on normal variation and the age of the fetus. The smooth lining of the bursa passes without any definite transition into a region that appears to be formed of very loosely-arranged connective tissue. The distal extremity is somewhat more definitely marked and usually is fairly distinct where it passes from the roof of the bursa, just beneath the deltoid, to the floor, which is fused with the articular capsule. However, in many cases, particularly in younger fetuses, the same sort of connective tissue is found in this region as far under the acromion. The medial or anterior and lateral or posterior walls are usually definite and the loose extra-mural connective tissue can easily be dissected away without injuring them. Superficially or above they reach the roof of the bursa, which is continuous with the precartilage of the acromion and the connective tissue under the deltoid muscle, and deeper or below they merge with the floor of the bursa.

In the early fetal period the bursa is almost wholly confined to the region beneath the acromial process. Figure 1 shows a bursa (x) from a 32.5 mm. fetus (CR). Above, it has extended to the region occupied by the precartilage of

the acromion and below practically to the dense connective tissue of the joint capsule. Loose connective tissue surrounds the rest of the bursal wall. Figure 2 is a higher magnification of the bursa shown in figure 1 and the loose connective tissue about the walls is more clearly seen.

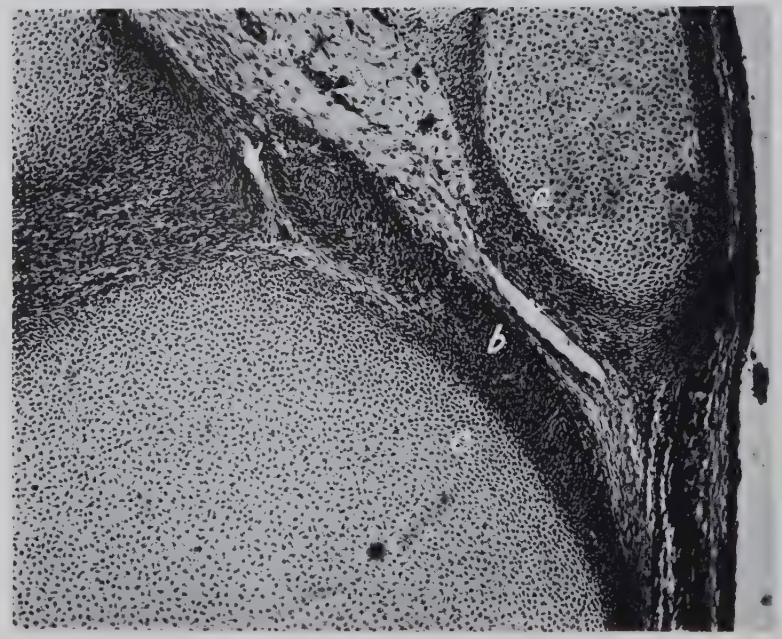
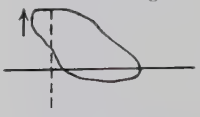


Fig. 1 Frontal section through the shoulder of a fetus. (a) the acromion; (b) the joint capsule and the tendon of the supraspinatus; (c) the head of the humerus; (d) the beginning of the articular slit; (x) a small subacromial bursa. The diagram below shows the form of the bursa from above. The dotted line indicates the position of the lateral margin of the acromion; the solid line, the plane of the section. The arrow points ventrad. CR—32.5 mm. Delafield's hematoxylin-eosin. × 80.



In somewhat larger fetuses, the bursae have extended beyond the acromion beneath the deltoid muscle, but there has been relatively little extension further under the acromion. Figure 3 shows this bursa in a 57-mm. fetus (CR). The proxi-

mal wall underneath the acromion is very poorly formed and the bursa appears to be multilocular. Two small loculi, the walls of which appear to be little more than loose connective tissue slightly compressed above and below, are seen at the right extremity of the bursa. The lining of the floor and roof, however, with the exception of that of the most medial part

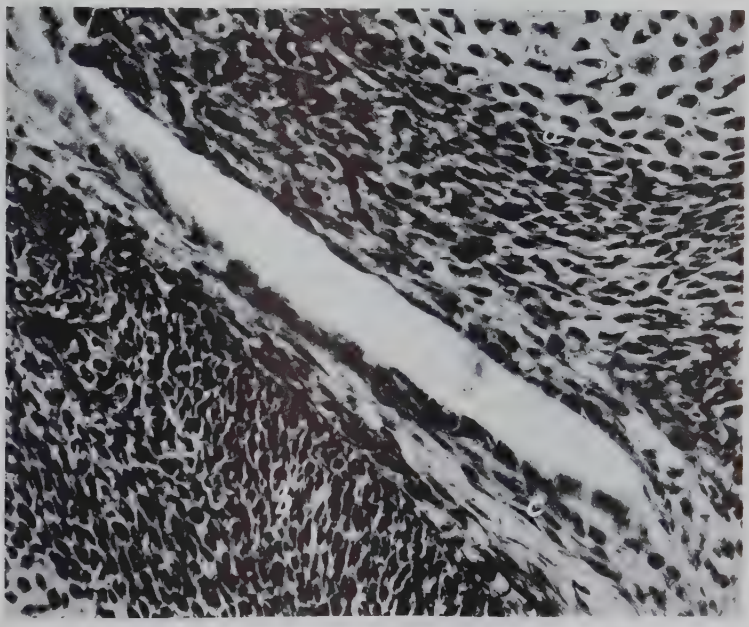


Fig. 2 Higher magnification of bursa seen in figure 1, to show the cells lining the bursa. (a) the acromion; (b) the joint capsule and the tendon of the supraspinatus; (c) connective tissue cells arranged parallel to the long axis of the bursa. CR—32.5 mm. Delafield's hematoxylin-eosin. × 360.

of the floor, appears to be slightly richer in cells than the surrounding tissue. In this figure a large trabecula composed entirely of cells with elongated nuclei can be seen extending completely across the bursae.

The smaller bursa lies almost wholly beneath the acromion, but the larger extends anteriorly and laterally under the

deltoid, although the form has changed little. This same general form is maintained, with slight elongation, even in full term fetuses.

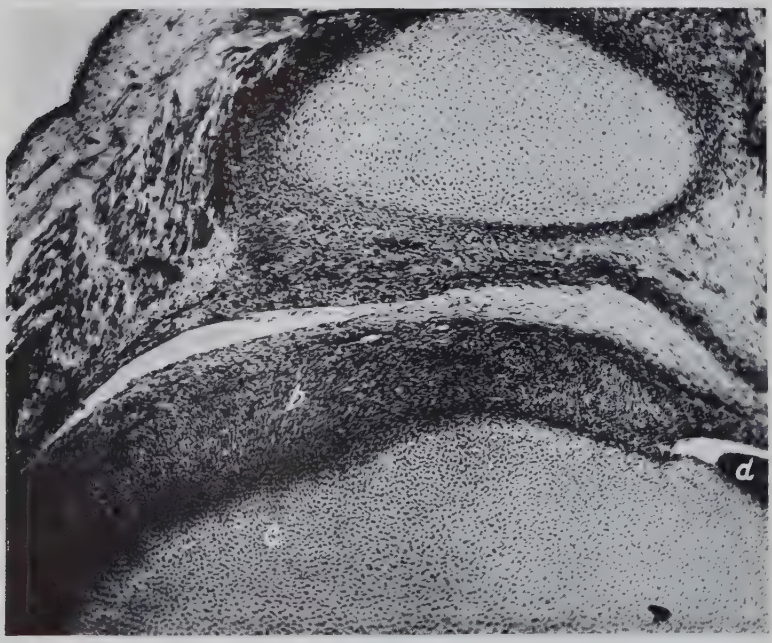
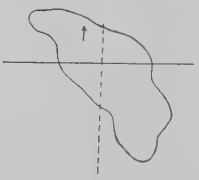


Fig. 3 Frontal section through the shoulder of a fetus. (a) acromion; (b) joint capsule; (c) head of the humerus; (d) tendon of the long head of the biceps; (e) joint cavity; and (f) deltoid muscle. (x) indicates a large bursa located partly under the acromion and partly under deltoid muscle. The diagram shows the form of the bursa from above. The dotted line indicates the position of the lateral margin of the acromion; the solid line indicates the plane of section. The arrow points ventrad. CR—57 mm. Delafield's hematoxylin-eosin. × 80.



The higher ratio of the length to the width of the bursa in older fetuses may be correlated with the structure of the walls. It was pointed out above that the proximal and distal walls of these somewhat rectangular bursae are indistinct and continuous with the loose surrounding connective tissue. The

proximo-distal extension of the bursa takes place at the expense of these walls. The inferior and superior walls very early approach their limits of extension, for the capsule of the shoulder joint, the acromion and the deltoid make any further extension in these directions impossible. Definite bursal walls are present medially (anteriorly) and laterally (posteriorly), at least in larger fetuses, and little further extension takes place during the prenatal period.

The structure of the walls of fetal bursae can be likened only very qualifiedly to that of adult bursae, if, as Key ('28) declared, adult synovial membranes should be classified according to the tissue on which they lie, for there would then be synovial membranes or strata of the areolar type, of the adipose type, and of the dense fibrous type, though all types of synovial membrane may be present in the same bursa. The types may also vary in the same region of the same bursa in different individuals. In fetuses, bursae are present before the collagenous fibers are well formed or very numerous. They are present before the fat is deposited in the superficial fascia and at a time when growth is rapid. Since this investigation was confined to fetal material, and the microscopic developmental study to one bursa, no discussion of these various types of bursal wall can be attempted, but a description of the very early fetal synovial membrane of the subacromial bursa will be given to illustrate its structure.

The cells on the surface of a very small, developing subacromial bursa are larger than the surrounding connective tissue cells and stain somewhat differently. This gives the effect of a specialized lining, particularly if the bursa has not extended to the joint capsule or the acromion. In figure 2 the difference between the lining cells and the ordinary connective tissue cells can be seen. The differentiated cells are not restricted to the bursal wall, however. They are found throughout this particular region, and are all arranged with axes more or less parallel to the long axis of the bursa. This orientation of cells creates the appearance of rather definite bounding walls, except where there is little to suggest the presence of either a definite wall or a specialized lining.

In somewhat older fetuses, the enlarged cells cannot be seen, except in those parts of the bursa that are obviously enlarging. For the most part, the cavity is lined by cells that are identical to the connective tissue cells that surround it. The orientation of the cells has not changed and hence the ends of these larger bursae also seem to lack a definite wall.

The presence and nature of a trabecula is shown clearly in figure 3. The trabecula is composed of variously oriented connective tissue cells and extends completely across the bursa. It seems to be a remnant of the originally continuous connective tissue and at the age shown is composed of cells only. Later, collagenous fibers develop in relation to these cells and then all of the essentials are present for the mature trabecula.

In bursae which form in response to use, the conditions for the development of trabeculae are somewhat different. Here both the cells and the collagenous fibers are present and for some reason, probably having to do with the intensity of the friction and pressure, part of this tissue disappears, leaving trabeculae. These may hypertrophy and the surface cells may increase to constitute a typical synovial surface which, however, may be destroyed by friction later on.

In the olecranon region, there is some danger of overlooking bursae if one depends entirely upon dissection, for they may consist of small loculi with indistinct walls which could easily be confused with spaces in loose connective tissue. On the other hand, it also is very easy in this region to mistake the loose connective tissue for a bursa, particularly after the seventh month, for by this time much fat has been deposited in the subcutaneous tissues about the olecranon but very little immediately over it. This comparatively fat-free tissue, especially in preserved specimens, is very loose and is saturated with fluid. If the skin is reflected, the appearance in the olecranon region is somewhat like that of a bursa. There is usually a small oval area completely surrounded by fatty tissue, and because of the breaking of the collagenous fibers in the removal of the skin and the subsequent disappearance

of these ends in the soft fat-free mass, the area appears in many cases to have a smooth, moist surface simulating a membrane. This may be the explanation for the discrepancy between the results of some other investigations and the present.

In the dissections on this series of fetuses, a method was used that was designed to reduce these chances of error as much as possible. Two incisions were made at right angles to each other over the olecranon and deep enough to cut into the cartilaginous process. The four quadrants of skin were then reflected singly by carefully cutting the strands of connective tissue (retinaculae cutis) that pass between the skin and underlying tissue. The four quadrants of subcutaneous tissue and superficial fascia were then similarly reflected. The advantage of this method over that of simply reflecting the skin over the whole area and then the underlying tissue is that after one quadrant of tissue has been reflected, a careful examination of undisturbed cross sections through other quadrants is possible.

There is much disagreement in the literature on the time of the appearance of the subcutaneous olecranon bursa. Velpeau (1843) believed that he found it in fetuses as early as the fourth month and Whittaker ('10), using methods of injection and dissection, found it in eight out of twenty full-term fetuses. On the other hand, Gruber (1857 and 1866) found no subcutaneous bursae earlier than the tenth year, and Heineke (1868) agreed that these bursae were not found until very late. Bize (1896) failed to find it in three fetuses 5 to 6 months old. Dömény (1897) did not find it in fetuses, and in a 2-month child he found only two small connective tissue spaces in this region.

In this series of 200 fetuses no subcutaneous olecranon bursae were found by dissection. Hence the soft tissues over the olecranon of six fetuses, varying in age from the fourth month to full term, were removed en bloc, embedded, cut and examined microscopically. No bursae were found in this series of sections nor were there any indications of their formation.

Figure 4 shows a section through the olecranon and the overlying tissues from a 7-month fetus. At this age the subcutaneous fat is being deposited, but the process is not yet complete. By the eighth month the subcutaneous fatty layer is well formed, except in a small area immediately over the most prominent part of the olecranon where no fat is seen. There is no suggestion of a bursa superficial to the tendon of the triceps at this age.

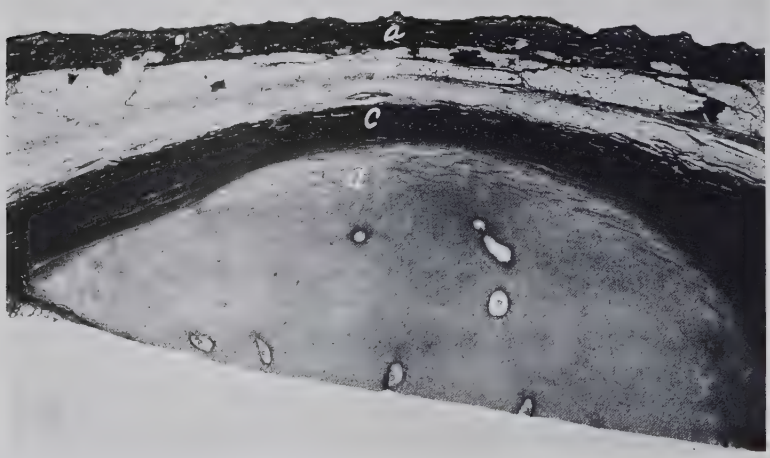


Fig. 4 Longitudinal section through the olecranon of a 7-month fetus showing the complete absence of any bursa in the soft tissues over this process. (a) skin; (b) the subcutaneous tissues and the superficial fascia. At this stage the subcutaneous fat is being deposited; (c) the tendon of the triceps; (d) the olecranon process. CR—about 250 mm. Delafield's hematoxylin-Mallory II. × 15.

Dissections in the prepatellar region of fetuses, to determine the presence of bursae, are no more reliable than those in the olecranon region, although the former region is larger. Instead of a relatively small rounded prominence, there is a larger flat one over which the bursa may be found. A definite layer of subcutaneous fat is found here in fetuses older than 6 months. This reduces the possibility of erroneously considering prepatellar connective tissue spaces as bursae or as their beginnings.

A consideration of the layers in the prepatellar region of the fetus seems advisable for the proper understanding of the presence of bursae. According to Poirier (1886), in the adult there is subcutaneous connective tissue almost completely without fat next to the skin. Next comes a loose superficial fascia and under this, an extension of the fascia lata, which passes in front of the patella and inserts into the

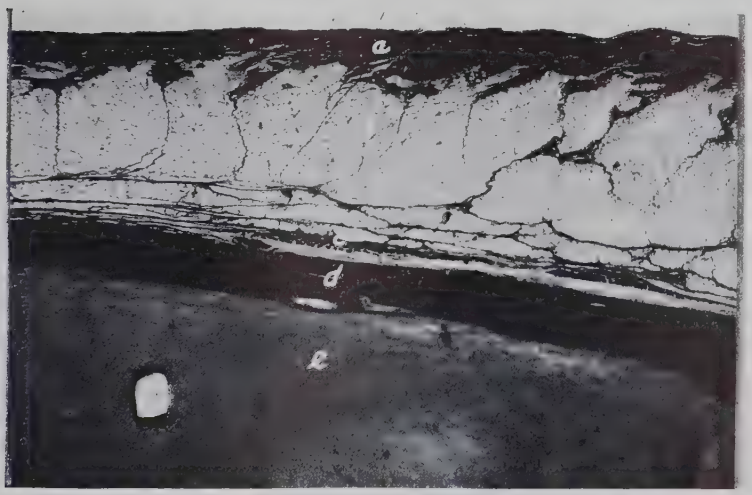


Fig. 5 Longitudinal section through the patella and prepatellar tissues of an 8-month fetus. The panniculus adiposus is well formed, but there is no indication of a bursa. (a) the skin; (b) the subcutaneous tissues; (c) the fascia lata; (d) fibrous mass formed in part by a portion of the tendon of the rectus and in part by expansions from the tendons of the medial and lateral vasti; (e) the patella. CR—about 270 mm. Delafield's hematoxylin-Mallory II. × 15.

capsule of the knee joint. Beneath this, there is another similar layer formed by fibers from the tendons of the two vasti crossing over the midline in front of the tendon of the rectus femoris. Finally, there is the patella, clothed by the most superficial fibers of the tendon of the rectus and the periosteum.

The layers in this region of a fetus are shown in figure 5. The subcutaneous connective tissue and the superficial fascia

are merged and loaded with fat. At (c) fibers of the fascia lata can be seen. Poirier considered that the most superficial of the three prepatellar bursae, the subcutaneous, was found between the superficial fascia and the fascia lata. At (d) is a tendinous mass fused with the patella which is formed partly by the tendon of the rectus and partly by expansions from the tendons of the vasti. In the adult, the subfascial prepatellar bursa is located underneath the deep fascia and the subtendinous or 'deep prepatellar bursa' (Luschka) beneath the expansions from the tendons of the vasti.

The prepatellar tissues in the fetus show no indications of bursae or the presence of bursal development. In fetuses older than 7 months, the fatty layer is somewhat thinner than the fat surrounding the patella, but its structure does not change over the entire region. In younger fetuses, too, there is no particular difference in the soft prepatellar tissue in any part of the region. The tissue between the fascia lata and the tendinous mass, in older fetuses, is very moist and loosely arranged. A similar method of dissection was used in this as in the olecranon region and the results were uniformly negative in the 400 fetal knees.

Any lingering doubt of the existence of bursae was removed when the prepatellar tissues of the six fetuses mentioned above were sectioned for microscopic study. This examination showed that the tissue between the tendons and the fascia lata is very loosely arranged and composed mostly of bundles of collagenous fibers that merge above with the fascia lata and below with the tendons. No cell-rich surfaces are found on any of these bundles.

In the fetus the expansions from the vasti are fused with the tendon of the rectus femoris and the patella and this fact precludes the possibility of the occurrence of the subtendinous bursa before birth.

There is as much disagreement in the literature concerning the time of origin of the prepatellar bursae as there is concerning that of the subcutaneous olecranon bursa. Velpeau (1843) thought that the subcutaneous prepatellar bursa de-

veloped at about the same time as the subcutaneous olecranon bursa. Bize (1896) found the subcutaneous prepatellar bursa well developed in two newborn. Dömény (1897) found no subcutaneous bursae in a 6-month fetus, but he found the subcutaneous and the subtendinous prepatellar bursae in older fetuses and in the newborn. He also believed that the subfascial bursa developed toward the end of intrauterine life and agreed with Moser (1892) that it was the first of the prepatellar bursae to form. On the other hand, Poirier (1886) believed that the superficial prepatellar bursa was not found in the fetus or in the newborn. Gruber (1857 and 1866) and Moser (1892) agreed that subcutaneous bursae are not present until the post-natal period.

Figure 5 is from a section of the prepatellar tissues of an 8-month fetus. There is no suggestion of a bursal cavity.

The anatomical relationships of the subacromial bursa are such that it can be found with some certainty by dissection in fetuses older than 3 months. After the deltoid muscle has been reflected, a fascial layer can be seen extending distally from the tip of the acromion. This is formed partly by the bursal wall and partly by the deep layer of the fascial sheath of the deltoid. An incision just underneath the tip of the acromion through this fascia will open up the bursa. If no bursa is present then, loose, irregularly-arranged connective tissue that can be separated with equal ease in all directions is found in this region. In younger fetuses the region is so small that dissection cannot be relied upon and in the small specimens preserved in toto, the moistness, color and texture of the subacromial tissue is such that bursae are very difficult to see.

Older fetuses usually have larger bursae in this region than do younger, but in some cases very small bursae are found even at term. In these cases very loose connective tissue is present in the areas where portions of a bursa are usually found. It is quite possible that some of the smaller of these bursae were missed in the dissection or that a small bursa was mistaken for a connective tissue space or artifact.

Whittaker ('10) was the only writer of those consulted who gave data on the incidence of the subdeltoid and subacromial bursae in the fetus. He found the subacromial bursa constantly present at birth in twenty specimens and it was multilocular in two specimens. He found a separate subdeltoid bursa in only one fetus out of twenty.

In my series of 400 fetal shoulders, 290, or 72.5 per cent, had a true subacromial, a subdeltoid, or a subacromial-subdeltoid bursa. This incidence is probably too low, for the chance of overlooking a bursa in gross dissection must be borne in mind and is much greater than the opposite chance of calling some artifact or connective tissue space a bursa. I considered all doubtful cases as negative and regarded a bursa as present only when it was a definite cavity surrounded by a somewhat specialized or oriented connective tissue. The cavity must have been large enough to avoid confusion of it with a connective tissue space.

In 208 of the fetal shoulders a subdeltoid-subacromial bursa was present; in 47, a subacromial only; and in 30, a subdeltoid only. Of the 200 fetuses, 130, or 65 per cent, had bilateral bursae; in 15 per cent it was unilateral; and in 20 per cent there was none in this region. In 13 of the 30 fetuses with a unilateral bursa it was present on the right side, while in 17 it was present on the left.

Five out of 200 fetuses had both a subacromial and a subdeltoid bursa on the same side. In three of these they were on the left, and in two on the right side.

The percentage of fetuses having bilateral subacromial bursae is quite similar in the different age groupings from the fourth through the ninth month (table 1), which indicates strongly that the majority of these bursae develop before the fourth month and not between the fourth month and birth. My findings on the series of twenty specimens the shoulders of which were embedded and studied microscopically, indicate that the subacromial bursa begins to develop in the early part of the third month. It is present in fetuses longer than about 35 mm. (CR) with practically the same constancy as

it is found in older material. In specimens between about 20 and 35 mm. (CR) the presence of bursae and anlagen of the same, indicates that it is at this time that these cavities first appear. In fetuses of this length, well-formed bursae are rather rare, but in most cases there is either an anlage

TABLE 1

Table 1 shows the distribution of the bursae in the different monthly age groups. Many dissections were attempted on fetuses in the third month, but because of the great uncertainty, all but three fetuses with easily recognizable bilateral bursae, were rejected. The three fetuses were retained to indicate that definite bilateral bursae, recognizable during gross dissection, do occur in this region in fetuses of this age, and to complete the monthly age groupings in the table

	3	4	5	6	7	8	9	TERM
Per cent of cases in which bilateral bursae were present	(100)	67	63	65	47	63	67	79
Per cent of cases in which unilateral bursae were present on right side	....	2	6.5	11.7	6	10.5	..	10
Per cent of cases in which unilateral bursae were present on left side	....	14	8.5	11.7	..	5.5	9	..
Per cent of cases in which bursa were bilaterally absent	....	17	22	11.6	47	21	24	11
Total of fetuses in each group	3	41	46	34	17	19	21	19=200

or a region in which slits have begun to appear in the connective tissue. In older material, from 35 to 55 mm. (CR), well-formed cavities, lined either with differently appearing cells or with no specialized lining, are usually found. In fetuses longer than 55 mm., a well-formed cavity or loose connective tissue is found in this region.

The appearance of the bursal wall in the latter part of the third month lends further weight to the supposition that these bursae usually form before this time. It was pointed out above that the cells forming the inner lining of bursal walls are structurally different in the newly-formed bursa (figs. 1 and 2) than in those that have been formed for some time (fig. 3). Fetuses in the latter part of the third month generally have bursal walls of this more mature form, while those from specimens in the early part of the month generally have a lining of cells with swollen, lightly staining nuclei.

Very definite bursae were found in this region in fetuses as small as 32.5 mm. (CR). One somewhat doubtful cavity was seen in an embryo of a (CR) length of 20.5 mm. This cavity, although small, was probably a bursa, because of the appearance of the cells making up the walls and because of its position. From the appearance and the number of such bursae present in very young fetuses, it is quite probable that they generally develop at a slightly later time and that they are very rare in embryos smaller than about 20 mm. (CR). The first appearance of these bursal cavities is closely correlated with the first appearance of a slit for the articular cavity of the shoulder.

The earlier writers on the formation of bursae generally agreed that these cavities were formed in response to use and that they developed as a result of the coalescence of pre-existing connective tissue spaces. They concluded that completely-formed bursae merely were enlarged connective tissue spaces with walls somewhat denser than the surrounding connective tissue. Henle (1838), Velpeau (1843), Malgaigne (1859), Richat (1866), Pineau (1866), and others were of this opinion.

Velpeau was one of the first to find bursae in the human fetus, but he considered that they had been formed by the friction attending fetal movements. Shortly after this time, however, bursae came to be regarded by some investigators as developing during fetal life independently of fetal movements, and this belief gradually became the more popular

near the end of the last century. Cruveilhier (1843), Heineke (1868), Debierre (1888), Retterer (1896), Dömény (1897), and Poirier ('01), all agreed that some bursae at least were embryonic or fetal in origin and not dependent on fetal movement.

Neither the olecranon nor the prepatellar subcutaneous bursae were found in the fetuses examined nor did the subcutaneous tissues of these regions show any changes indicative of their formation. The chance of missing all of the bursae, if they were at all well formed, would be very slight even if no other method than careful gross dissection were used. Hence I feel justified in concluding that these subcutaneous bursae do not begin to form until after birth. The olecranon and the superficial prepatellar bursae are probably the most constant of the subcutaneous bursae in the adult, and the conditions that lead to their formation are probably as constant as those in any other region. For these reasons the negative results regarding them probably can be applied with some assurance to the subcutaneous bursae generally and, if so, it seems doubtful whether any subcutaneous bursae develop before birth.

The deeper prepatellar bursae also were absent in the fetus; the tendinous expansions from the vasti were fused so intimately with the patella that no space existed between them. However, it is possible that these expansions may separate from the tendon of the rectus and the patella at some postnatal time in consequence of motion, as Poirier holds, and a bursa thus form. Although the subfascial prepatellar bursa was not found in this series of fetuses, the appearance of the subjacent tissues suggests that it is probably the first to develop in the region.

In many younger fetuses, a well-defined mass of cells can be distinguished immediately under the tip of the acromion. In figure 6 a cellular mass is seen to occupy the region which in slightly older specimens is occupied by the bursal cavity. It extends upward to the precartilage of the acromion and downward to the joint capsule and gradually merges with the

subacromial or subdeltoid connective tissue on all sides. The cells of this mass are elongated and are arranged in a direction parallel to the long axis of the future bursa. The nuclei are large and stain less intensely than do the nuclei of the surrounding connective tissue cells. In fetuses of approxi-

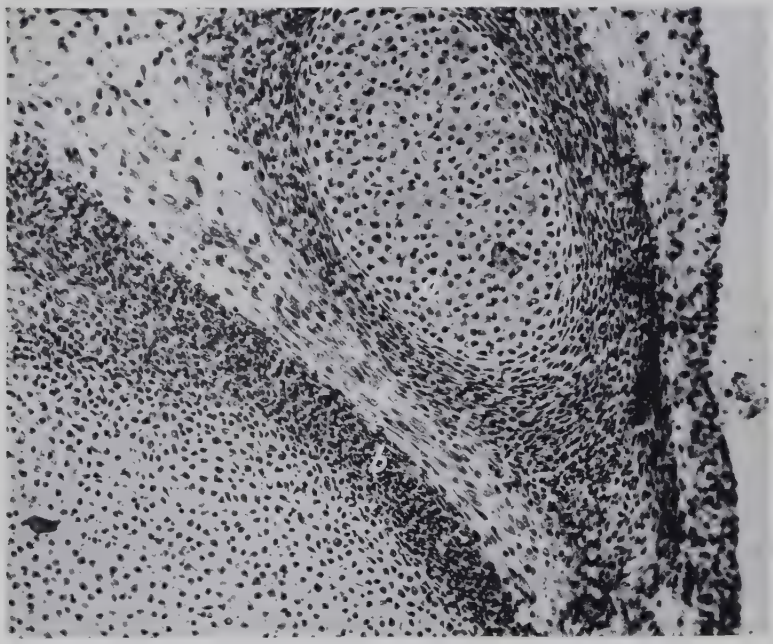


Fig. 6 Frontal section through the shoulder of a fetus, showing the arrangement of the cells before the appearance of a bursal cavity. (a) acromion; (b) joint capsule and tendon of the supraspinatus; (c) head of the humerus. (x) is in a region occupied by elongated cells with poorly staining nuclei. They are arranged predominantly parallel to the long axis of the future bursa. CR—28.5 mm. Delafield's hematoxylin-eosin. × 175.

mately the same age or very little older, slits begin to appear between the characteristically arranged cells. In figure 7 one of the earliest of these slits can be seen in the region (x). Very soon after the original slit, others appear near it, and the tissue between two slits, formed almost entirely of differentiated cells, either remains as a trabecula or soon dis-

appears during the formation of the cavity. The slits seem to form easily in the direction of the long axis of the bursa and appear to extend in a direction parallel to the cells of the anlage; so that in some cases the slits extend completely through the cell mass. However, the rows of cells seldom

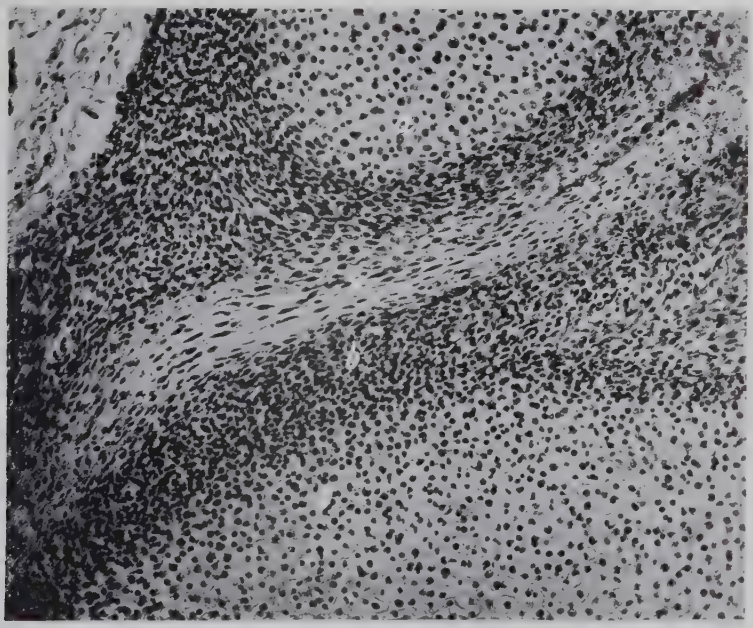


Fig. 7 Frontal section through the shoulder of a fetus showing in the region (x) the first appearance of the bursal cavity. A slit is forming between the elongated cells designated (x). Other lettering as in figure 6. CR—33 mm. Delafield's hematoxylin-eosin. × 175.

are parallel, and adjacent slits usually merge with each other to form wider cavities rather than extend entirely across the cell mass. The long, narrow cavities suggest that definite resistance is offered in a transverse plane to the widening of the slit.

Retterer thought that an open fiber meshwork formed before the development of the cavity, but the fibers, at least in

this location, are very fine and probably quite unimportant in the formation of the cavity.

Figure 8 shows this bursa in an older fetus. The loculi, and particularly that marked (x), appear to be lined by a condensed layer of cells. Some time after the longitudinal

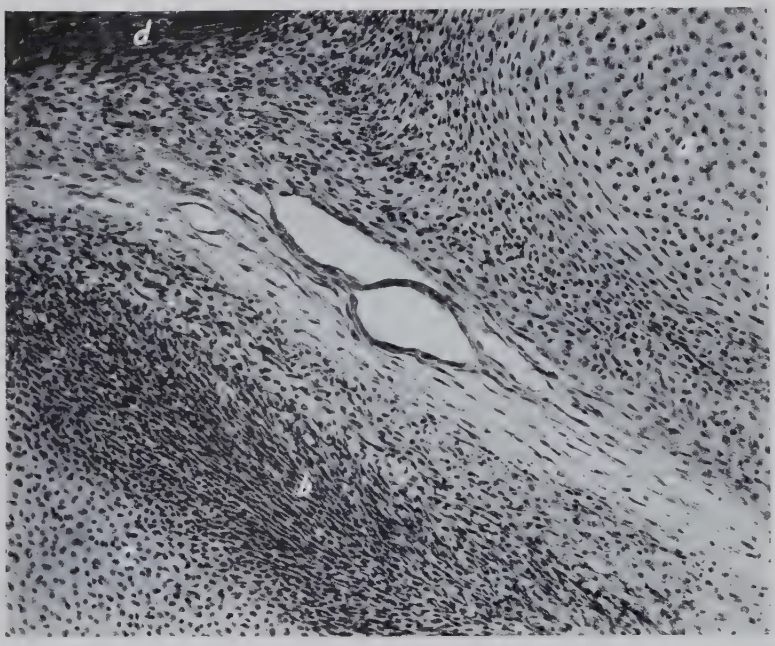


Fig. 8 Frontal section through the shoulder of a fetus, showing at (x) a bursal cavity, the walls of which are for the most part formed by elongated cells similar to those seen in the two preceding figures. Other lettering as in figure 6. CR—39 mm. Delafield's hematoxylin-eosin. × 175.

slits have appeared, the cell layers begin to disappear. This continues until all of them have disappeared, excepting the most peripheral which remain for a varying period when they, too, disappear. Figure 3 shows a bursa after these cells have disappeared. The formation of this bursa may be briefly summarized as follows:

The connective tissue of a fairly circumscribed area undergoes certain changes to form a fairly definite anlage composed of large, moderately closely packed cells which are arranged more or less parallel to the long axis of the future bursa. Slits appear between the cells and merge or remain adjacent. These slits extend throughout the bursal anlage, but stop at the border of the undifferentiated connective tissue. The early slits develop into a definite cavity lined by swollen, characteristic cells. When the cavity attains a certain size, the lining cells disappear and the cavity comes to have a lining of ordinary, but specially arranged, fibroblasts and other connective tissue elements.

SUMMARY

Since neither the subcutaneous olecranon bursa nor the subcutaneous prepatellar bursae were present in fetuses, it is probable that subcutaneous bursae do not develop before birth.

Neither the subfascial nor the subtendinous prepatellar bursae are present in the fetus, although the region in which the subfascial bursa is located is filled with very loose tissue. The latter probably develops before the other prepatellar bursae.

The subacromial bursa is present in the fetus. It develops early in the third month from a fairly definite anlage. The incidence of this bursa in the fetal period is not so high as in the adults. It was found to be 72.5 per cent before birth, when other factors may play a role in their formation.

The structure and form of the subacromial bursa in the fetus differs from that in the adult. It is more rectangular and does not extend relatively as far either posteriorly or anteriorly.

This investigation was undertaken at the suggestion of Doctor Meyer. I wish to express my appreciation to him for the privilege and for suggestions and assistance.

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MORPHOLOGICAL STUDIES ON THE ANTERIOR PITUITARIES OF MATURE FEMALE RATS RECEIVING INJECTIONS OF PREGNANCY URINE EXTRACTS [1]

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TWO PLATES (THIRTY-SIX FIGURES)

It was reported by Wolfe, Phelps and Cleveland ('33) that injection of human placental extracts, rich in the A. P. L. factor, brought about marked changes in the anterior pituitaries, as well as in the ovaries of immature female rats. The ovaries of the injected animals were markedly increased in size, while the pituitaries showed a moderate increase in weight over the controls. The pituitary weight increase was usually greatest in animals in which the ovarian reaction was greatest. Histologically, the pituitaries of the injected animals were markedly altered from the controls. The most outstanding changes were in the basophiles; these cells were increased in size, but exhibited marked loss of granular material. Many of the eosinophiles were swollen and also showed evidence of granular loss. Cell counts made on complete serial sections revealed the fact that the percentage of the eosinophiles was decreased, while that of the chromophobes was increased. Of equal significance was the fact that if the animals were castrated at the beginning of the

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injection period, the placental extracts had no effect on the anterior hypophysis.

Severinghaus ('34) has reported that injections of pregnancy urine extracts into mature female rats induced an increase in the number of the basophilic cells, but that they exhibited evidence of a rapid atypical granular depletion. He found that such extracts had no effect on the pituitaries of castrated rats. Wolfe, Ellison and Rosenfeld ('34 a and '34 b) reported that injections of extracts of pregnancy urine induced marked changes in the anterior pituitaries of normal female rats; particularly notable was loss of granules from both the basophiles and the eosinophiles. However, such extracts were without action on the pituitaries of castrated female rats. Wolfe ('34) later pointed out that such extracts induced similar changes in the anterior pituitaries of immature female rats. In this paper we wish to present certain of our studies on the effect of extracts of pregnancy urine on the anterior pituitaries of normal female rats.

MATERIAL AND METHODS

In these studies forty-four mature female rats received subcutaneously from 25 to 75 rat units of pregnancy urine extract [3] daily for 15 days. Vaginal smears were made daily throughout the experimental period. At sacrifice the ovaries and pituitaries were weighed on a torsion balance (Hartmann-Braun, accurate to 0.5 mg.). To ascertain the weight of the hypophysis in relation to the body weight, a factor was derived by dividing the pituitary weight by the body weight and multiplying the result by a suitable constant. The ovaries and the accessory reproductive organs were fixed in Bouin's fluid and stained with hematoxylin and eosin. The pituitaries were fixed in Regaud's fluid and prepared for study by methods already described (Cleveland and Wolfe, '32). Cell counts were made on all pituitaries studied.

[3] The Follutein which was used in these experiments was furnished by E. R. Squibb & Sons through the courtesy of Dr. J. J. Durrett.

The recent studies of Wolfe and Cleveland ('33), in which they described cyclic histological changes in the anterior hypophysis of the rat, correlated with the various phases of the oestral cycle, were used as controls. To date we have studied and made cell counts on the anterior pituitaries of sixty-nine female rats killed during the normal oestral cycle. In this series the mean level of the eosinophiles was 33.6 per cent, with a standard deviation of 4.5. The mean level of the basophiles was 4.8 per cent, with a standard deviation

TABLE 1

Data from forty-four injected rats are presented in this table. The animals are grouped according to the weight of the ovaries

NUMBER OF RATS	OVARY WEIGHT RANGE	AVERAGES				PERCENTAGE OF CELL TYPES PRESENT				CELLS COUNTED
		Ovary weight	Body weight	Pituitary weight	Pituitary factor	Eosinophiles	Basophiles: Granular	Basophiles: Non-Granular	Chromophobes	
	mg.	*mg.*	*mg.*	*mg.*						
3	100–150	126	170	9.2	5.4	28.2	—	1.8	70.0	15,071
14	151–200	177	180	9.9	5.5	28.3	0.6	2.1	69.0	57,999
9	201–250	229	185	11.9	6.4	27.3	0.2	1.4	71.1	40,010
7	251–300	276	218	14.8	6.8	25.2	0.2	1.5	73.1	24,480
2	301–350	341	220	13.3	6.1	22.3	0.2	2.3	75.2	9,518
3	351–400	374	222	16.5	7.4	22.7	0.1	0.5	76.7	17,342
2	401–450	418	239	18.5	7.7	22.9	0.2	0.4	76.5	7,729
3	501–550	508	210	15.9	7.5	20.6	0.4	0.4	78.6	15,240
1	750–800	766	238	26.0	10.9	14.0		0.2	85.8	5,954
									Total	198,343

of 1.2. The mean level of the chromophobes was 61.9 per cent, with a standard deviation of 4.2.

The effect of the injection of pregnancy urine extracts in normal female rats was constant from a qualitative viewpoint, but was variable quantitatively. There was a marked increase in the weight of the ovaries which varied from 106 mg. to 766 mg. (table 1). The ovaries of 137 normal female control rats varied from 39 to 84 mg., with an average of 58.6 (unpublished data). The injection period of 15 days was constant, but the daily amount of injected material varied from 25 to 75 rat units. Within these limits, the amount of

extract injected had no effect on the final weights of the ovaries; 25 units were as effective as 75. At autopsy, the ovaries of the injected rats were seen to be made up of tremendous numbers of very large corpora lutea and follicles, some of which were cystic. The weights of the pituitaries were also markedly increased; they varied from 8 to 26 mg., while the average pituitary weight of the controls, obtained from data on 137 normal female rats, ranged from 7 to 14 mg. (average of 10.8). Within certain limits, the increase in pituitary weight was greatest in animals in which the increase in ovary weight was greatest; this was most evident in animals in which the ovaries weighed over 200 mg. From table 1 it will be seen that the average pituitary weight of animals whose ovaries ranged in weight from 100 to 150 mg. was 9.2, with a pituitary factor of 5.4 (the average factor for normal female rats was 5.1). It can be seen from table 1, that, as the weight of the ovaries increased, there was a corresponding increase in the pituitary weights and factors. One animal (R-979), whose ovaries weighed 766 mg. at autopsy, had a pituitary weighing 26 mg.; the factor was 10.9.

Histological examination revealed that the anterior pituitaries of the injected rats were markedly altered from those of normal females. The glands as a whole were extremely hyperemic and edematous. Many of the eosinophiles, chromophobes, and practically all the basophiles [4] were swollen. These cells exhibited various degrees of granular depletion. The percentages of the granular cells, both eosinophiles and basophiles, were decreased, while that of the chromophobes was increased. One point should be emphasized; the morphological changes induced in all these glands were qualitatively

[4] In previous studies in which we described cyclic histological changes in the anterior hypophysis of the dog, the basophilic cells were subdivided into two types. For that reason we adopted a new terminology for the cell types of the anterior hypophysis. The eosinophiles were classified as cells of type I, the two basophilic types as II and III, respectively, and chromophobes as type IV. In subsequent studies carried out on rats, sows, guinea pigs and humans, only one basophilic type has been found. Therefore, in studies made on material from these species, the usual terminology of eosinophile, basophile and chromophobe will be used.

the same, but they varied in degree. Almost invariably the anterior pituitaries of rats in which the increase in the weights of the ovaries and pituitaries were greatest exhibited the most marked histological changes.

The basophiles exhibited marked granular depletion. In normal non-pregnant rats, the basophiles usually make up from 4 to 6 per cent of the total cells present. In some instances (dependent on the phase of the oestral cycle) they were well filled with granular material (figs. 1, 2 and 29); in others they exhibited partial or total granular loss (figs. 3, 4, 5, 6, 30, 31 and 32). In the greater number of all the basophiles a colorless or clear canal, in the form of an incomplete circle, was observed (figs. 1 through 7, 29 30 and 31). Supposedly, this represented the 'saftkanälchen' of Holgren, which is now considered by many investigators to represent the negative image of the Golgi apparatus. This apparatus in the basophiles was usually found between the nucleus and the cell membrane. Its size varied with that of the cell; in proportion to the size of the cell, it seemed of about equal size in the granular and non-granular forms. The basophilic cells lost their granular material and gave rise to a somewhat larger cell, with a light blue cytoplasm (figs. 5, 6, 30, 31 and 32). Our studies indicate that this cell eventually underwent regressive changes (figs. 7, 30, 31 and 32) and passed into the chromophobe.

In the anterior pituitaries of the injected rats, the level of the basophiles was markedly reduced. This was equally true in all the animals (table 1). Scarcely any completely granular cells were seen; most of those present exhibited complete or partial granular loss (figs. 3 through 7).

Changes in the eosinophiles were equally striking, but more difficult of analysis. In normal rats, these cells measured from 6 x 6 to 10 x 10 μ. They were usually well packed with granules which stained intensely with orange G and were usually round or oval in shape. In many of these cells the negative image of the Golgi apparatus was visible, usually in the form of a cap near the nucleus (figs. 10, 21, 22 and 23).

The eosinophiles of female rats killed during the various phases of the oestral cycle do not usually exhibit any marked evidence of granular loss (Wolfe and Cleveland, '33 a). However, Wolfe and Cleveland ('33 b) have reported that induction of a lutein phase into the cycle of the rat does result in such granular loss from these cells (figs. 11, 12, 13, 23, 24, 25 and 26). These cells, after granular depletion, gave rise to chromophobes. Reference to table 1 will show that in the rats receiving pregnancy urine extracts the level of the eosinophiles was reduced. Furthermore, the level of the cells was reduced more or less in proportion to the extent in which the ovaries and pituitaries were increased in weight (table 1).

The eosinophiles exhibited moderate to extreme degranulization. The number of these cells showing partial or almost complete degranulization was the greatest in animals in which the percentage of the eosinophiles was most markedly reduced. The cells showing any degree of granular loss were usually swollen and the remaining granular material took a lighter stain. In such cells the granules were more loosely packed throughout the cell, and in certain areas might be missing entirely (compare figs. 9 and 10 with 11, 12 and 13, and figs. 21 and 22 with 23, 24, 25, 26 and 36). In the cells which were completely filled with granules, the negative image of the Golgi body was usually rather small and closely applied to the nucleus (figs. 7, 8, 21 and 22). In cells which exhibited varying degrees of granular loss, the negative image of the Golgi body was often, but not invaribly, increased in size, sometimes to an extreme degree in the cells in which the granular loss was extreme. It was usually in most instances more or less crescented and situated close to the nucleus. The size and shape of the negative image of the Golgi apparatus may best be understood by studying figures 9 through 16 and figures 21 through 26 and 36. In some cells in which there was only a partial depletion of the granules, the remaining granules were less closely spaced and took a lighter stain with orange G and in certain portions of the cell were entirely missing (figs. 9 through 13, and figs. 21 through 26 and 36).

In many of the eosinophiles, the granular depletion was extreme (figs. 14, 15, 16, 27 and 28). These cells were enlarged and swollen. The negative image of the Golgi apparatus was also enlarged and was usually, but not invariably crescented about the nucleus (exception, fig. 27). In these cells only a very few pale yellow granules were seen, but a more homogeneous mass which took a pale yellow stain with orange G persisted in the region of the negative image of the Golgi apparatus. In the rest of the cell the cytoplasm was usually a light blue, which varied to a denser and darker blue on the periphery of the cell (figs. 16 and 28).

The chromophobes were by far the most abundant of the cells. They varied from 65 to 70 per cent in the animals whose ovaries weighed from 100 to 200 mg. and became more abundant in the pituitaries of rats which presented heavier ovaries. In one rat (R-979), whose ovaries weighed 766 mg., and pituitary, 26 mg., the chromophobes made up 85.8 per cent of the total cells present. This gland was of particular interest because of the extreme dilatation of the capillaries. The cells were separated into widely divided cords (fig. 20), much like the condition found in the pituitaries of pseudopregnant rabbits. These cords consisted almost entirely of chromophobes. For the rat this is decidedly unusual and is the only time we have ever observed it to this extent.

The appearance of the chromophobes varied somewhat. In some instances they were extremely large cells with a light grayish blue cytoplasm, which was almost colorless. In some such cells the negative image of the Golgi apparatus was very large (figs. 17, 33 and 34). In other instances, the nucleus was surrounded by fragmentary cytoplasm, almost colorless, and the negative image of the Golgi body was faint (fig. 18). Many of the chromophobes were smaller cells, with a deep blue cytoplasm; in such cells the negative image of the Golgi varied in size (figs. 19 and 35). In several previous papers we have reported that the granular cells undergo granular depletion and give rise to chromophobes. The studies presented in this paper confirm this view. Furthermore, it seems

highly probable that the chromophobes in turn give rise to new granular cells. The pituitaries of these animals were similar to those of rats killed in early pregnancy and pseudopregnancy, in that there was a loss of granules from both the basophiles and eosinophiles. In fact, the anterior pituitaries of the animals whose ovaries weighed up to 200 mg. and in which the level of the eosinophiles was above 25 per cent were practically identical to those of rats killed on the fifth or sixth day of pregnancy. However, in the experimental rats whose ovaries and pituitaries had been stimulated to a greater weight, they differed in a quantitative sense; the loss of granules from the granular types was more marked and involved a greater proportion of the cells, especially of the eosinophiles, since the basophiles were practically depleted in all the glands. It seemed that in this group of glands we were dealing with an intensification of the changes which normally occur in early pregnancy or pseudopregnancy. Such loss of granular material from the granular types has been considered indicative of an active secretory process.

Colloid was found in the residual cleft of thirty-eight of the forty-four injected animals. Such colloid is also found occasionally in the pituitaries of normal rats killed in different phases of the oestral cycle, but never in such a high percentage. Mitotic figures were also abundant (fig. 8). They were found in thirty-three of the forty-four glands studied. Each section studied and on which cells counts were made was examined closely and each mitosis recorded. The number counted per gland varied from 1 to 87. Mitotic figures were usually more abundant in animals in which the ovaries and pituitary weights were greatest, but this was not a constant finding. Mitoses were most abundant in the chromophobes, but were found also in the eosinophiles. We have never observed them in the basophiles.

DISCUSSION

Severinghaus ('34) has reported that injections of pregnancy urine extracts into non-castrated rats resulted in an increase in the basophiles, but that these cells underwent atypical degranulization. Their cytoplasm appeared as clumped yellowish masses interspersed with basophilic granules. The Golgi apparatus was hypertrophied. In our studies we have also found a granular depletion of the basophiles, but, in contrast to the findings of Severinghaus, we found a decrease in the percentage of these cells. The technical methods used in these experiments do not give a positive image of the Golgi apparatus, but they do give an excellent negative image which is similar to that recently pictured by Severinghaus ('34). Our studies indicate that, while the negative image is usually larger in the basophiles which exhibit loss of granules, the cell itself is enlarged, and that the size of the negative image of the Golgi apparatus is not definitely out of proportion to the size of the cell.

In our studies we have also reported changes in the eosinophiles which were most marked in rats exhibiting greatest weight increases in the ovaries and the pituitary. These cells exhibited granular depletion, accompanied by an increased size or swollen condition of the cell. In a greater number of those cells exhibiting granular depletion, there was an increase in the size of the negative image of the Golgi apparatus. In such cells the granules had either partially or totally disappeared, although in most a yellowish mass tended to persist in the region of the Golgi apparatus. The percentages of the eosinophiles were moderately or extremely reduced (dependent on the size of the ovaries).

In previous studies on changes occurring in the anterior hypophysis during the different phases of the oestral cycle, we found changes in both the basophiles and the eosinophiles, but the changes in the eosinophiles were limited to that phase of the cycle in which active corpora lutea were in the ovaries. In the early lutein phase there was loss of granules from and a decrease in the percentages of the eosinophiles, as well as

marked granular depletion of the basophiles. In the group of experimental rats considered in this paper, a tremendous number of very large corpora lutea were induced. Associated with this was a marked weight increase in the pituitary and a marked loss of granules from the eosinophiles of the anterior lobe. In this group of rats it appeared that we were dealing with an exaggeration of the changes which normally occur in early pregnancy or pseudopregnancy in the rat, i.e., marked granular depletion of the basophiles and a moderate granular loss from the eosinophiles. This granular loss was intensified in this group of experimental animals in which the weights of the ovaries were increased many times when compared with those of normal rats.

The marked variation in the weight response of the ovaries and the pituitaries presents a problem for which a solution is at present not forthcoming (table 1). The injection period was the same in all cases; i.e., 15 days. The daily dosage of pregnancy urine extract varied from 25 to 75 rat units, but, as stated before, this was not responsible for the varied response. It would seem that there was possibly a depletion of a certain factor in the complex of the animal's own pituitary and ovaries and the factors present in the extract of pregnancy urine.

SUMMARY AND CONCLUSIONS

Injections of pregnancy urine extracts into normal female rats for a period of 15 days resulted in marked weight increases in the ovaries and the pituitaries. The weight increase in both organs was variable, but the pituitary weights were greatest in rats exhibiting the heaviest ovaries. Histological examination of the pituitaries revealed an extreme granular depletion of the basophiles and a less marked depletion of the eosinophiles. Both types were reduced in percentage while the chromophobes were increased. Such changes are considered indicative of secretory activity. Depletion of the granules of the basophiles and the eosinophiles, and the reduction in the percentages of these cells was most marked

in rats in which the weight increase in the ovaries was greatest. Colloid was found in the residual cleft of practically all the injected rats. Mitotic activity was marked. Cell counts were made on all glands studied.

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PLATE 1

EXPLANATION OF FIGURES

1 A small granular basophile. A faint negative image of the Golgi apparatus is shown. G filter. × 1066.

2 A granular basophile of moderate size. The negative image of the Golgi apparatus is shown. G filter. × 1066.

3 A basophile exhibiting moderate granular loss. The negative image of the Golgi apparatus is shown, although it is faint. G filter. × 1066.

4 A basophile showing marked granular loss. The granular material remaining is clumped to left of nucleus. The negative image of the Golgi apparatus is shown. G filter. × 1066.

5 A basophile exhibiting total granular loss. The negative image of the Golgi apparatus is shown. G filter. × 1066.

6 A basophile exhibiting total granular loss. The light blue cytoplasm is slightly fragmentary. G filter. × 1066.

7 A basophile showing regressive changes. The light blue cytoplasm is markedly fragmentary. A faint negative image of the Golgi apparatus is shown. G filter. × 1066.

8 Mitotic figure in a chromophobe. G filter. × 1066.

9 Two small granular eosinophiles. In the cell to the right (see arrow) the negative image of the Golgi apparatus is shown. B filter. × 1066.

10 A large eosinophile filled with granules. The negative image of the Golgi apparatus is shown. B filter. × 1066.

11 An eosinophile showing slight granular loss. The slightly enlarged negative image of the Golgi apparatus is capped about the nucleus. B filter. × 1066.

12 A large eosinophile showing thinning of granules which are not well indicated because of filter used. See figure 36 for camera lucida drawing of same cell. G filter. × 1066.

13 An eosinophile of moderate size, showing a generalized thinning of granules. A portion of the negative image of the Golgi apparatus is shown capped on the nucleus. B filter. × 1066.

14 An eosinophile showing extreme granular loss. A few granules taking a pale stain with orange G were found in the region of the Golgi apparatus, the negative image of which is shown. G filter. × 1066.

15 An eosinophile exhibiting extreme granular loss. A homogeneous mass staining with orange G was found in the region of the negative image of the Golgi apparatus. G filter. × 1066.

16 An eosinophile showing extreme granular loss. A homogeneous mass staining with orange G was found in the region of the negative image of the Golgi apparatus. The peripheral cytoplasm was stained deep blue. G filter. × 1066.

17 A large chromophobe. The negative image of the Golgi apparatus is shown. The cytoplasm was pale gray. G filter. × 1066.

18 A chromophobe with pale grayish-blue cytoplasm. G filter. × 1066.

19 A chromophobe with a rather dense blue cytoplasm. The negative image of the Golgi apparatus is shown. G filter. × 1066.

20 Taken from rat 979 (see text). The capillaries are markedly distended. The cell cords are made up almost entirely of chromophobes. G filter. × 327.

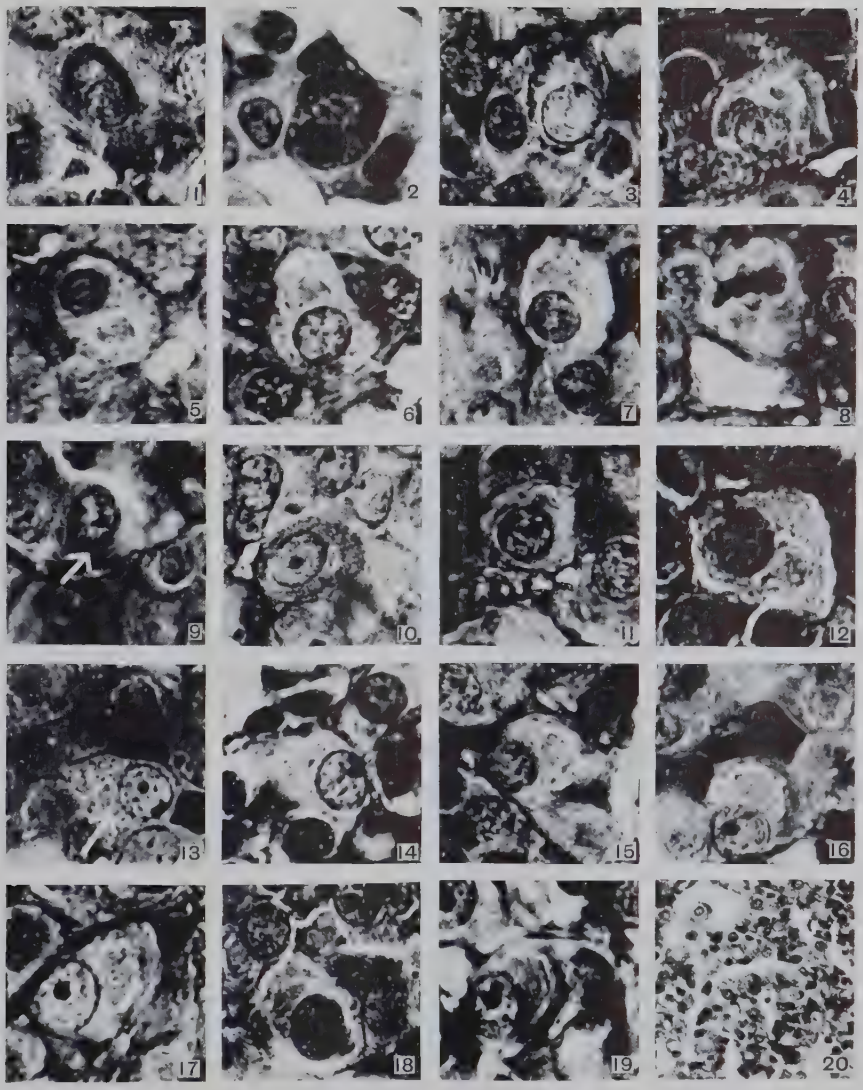
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PLATE 2

EXPLANATION OF FIGURES

These camera lucida drawings were made by Miss Roberta Cecil, of the department of illustration of Vanderbilt University School of Medicine.

21 An eosinophile complete filled with granules. The negative image of the Golgi apparatus is shown capped on the nucleus. × 1466.

22 An eosinophile showing the negative image of the Golgi apparatus closely applied to the nucleus. × 1466.

23 A larger eosinophile in which the granules are less closely packed and the negative image of the Golgi apparatus is slightly enlarged. This cell is also shown in figure 11. × 1466.

24 An eosinophile which is swollen and shows partial granular loss. The remaining granules took a pale stain with orange G. Near the upper pole of the cell the cytoplasm was dark blue. × 1466.

25 An eosinophile similar to that shown in figure 4, except that there is greater loss of granules. Notice negative image of the Golgi apparatus capped on the nucleus. × 1466.

26 An enlarged eosinophile showing almost complete granular loss. The cytoplasm in the lower part of the cell was dark blue. × 1466.

27 An eosinophile showing extreme granular loss. A yellow, slightly granular mass was found in the region of the negative image of the Golgi apparatus which, in this instance, was not capped on the nucleus. The rest of the cytoplasm was a very light blue. × 1466.

28 An eosinophile showing extreme granular loss. Yellow staining material persisted in the region of the negative image of the Golgi apparatus. This cell is also shown in figure 16. × 1466.

29 A granular basophile of moderate size. This cell is the same as that pictured in figure 2. × 1466.

30 A large basophile showing complete granular loss. This is the same cell as that pictured in figure 5. × 1466.

31 A large basophile showing a faint image of the Golgi apparatus and clumps of granular material. × 1466.

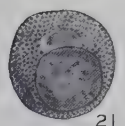
32 A large basophile entirely devoid of granules. The light blue cytoplasm is fragmentary. × 1466.

33 A large chromophobe with very pale grayish-blue cytoplasm. This cell is also shown in figure 17. × 1466.

34 A large chromophobe with pale grayish cytoplasm. The negative image of the Golgi apparatus is shown. × 1466.

35 A smaller chromophobe with dense blue cytoplasm. The negative image of the Golgi apparatus is shown. × 1466.

36 A large eosinophile showing thinning of the granules. The negative image of the Golgi apparatus is shown. The same cell is shown in figure 12. × 1466.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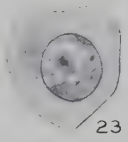
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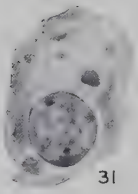
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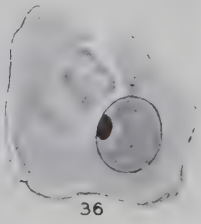
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BOOK REVIEW

FRANKLIN PAINE MALL; THE STORY OF A MIND. By FLORENCE RENA SABIN. Baltimore, The Johns Hopkins Press, 1934. ix + 342 pp., 8 full page illustrations.

It is now 17 years since the death of Franklin Paine Mall; the anatomical class rooms and even the junior ranks of the anatomical profession are occupied by young people who did not know him, and who are coming up in the well-equipped laboratories and the sound professional atmosphere of modern American medicine without realizing their debt to men like Mall who spent their careers in making our present opportunities for us. This story of Mall's life, written by one of his most distinguished students, Dr. Florence Sabin, will not only be treasured by those who came under his personal influence, but it will take its place almost as a guide-book for those newcomers to anatomical investigation and those students of medicine who desire an understanding of their own scientific environment.

One measure of a man's importance is the breadth of the stage which must be set for the drama of his life. To place Mall against his full setting Miss Sabin has had to sketch the whole movement for reform of medical education in the nineteenth century, for he began the study of medicine when our schools were still provincial and backward, and when the preclinical sciences were entirely in the hands of practitioner-teachers. To show, in turn, the full meaning of the 2 years spent in Leipzig, when he became a favored pupil and lifelong friend of Wilhelm His and of Carl Ludwig, requires the retelling of a great chapter in the source-history of American culture, and one which it is well to recall in these days of misunderstanding between nations. The series of letters from Ludwig to Mall, published for the first time in this book, not only affords a charming picture of the old-time German Gelehrter, but sets us an example of how one of the greatest teachers of all time gave himself heart and soul to a student for whom he foresaw an important future.

From this experience of the best in medical science, Mall returned to America to be one of the group of men similarly trained who were to reorganize our medical schools, set up modern research, and found our scientific journals. To explain his part in this move-

ment has not been an easy one for his biographer, for Mall was strangely different from the usual person gifted with 'leadership.' A man of great independence and originality, at once an idealist and a good practical executive, he was shy, reserved with strangers, and either unable or unwilling to speak in public. His teaching, both of the medical classes and of the group of advanced students who quickly gathered around him at Johns Hopkins, was patterned largely after the well-known (apochryphal) formula for bathing the baby attributed to Mall by the students and here retold by Doctor Sabin: "Put her in the tub and let her work out her own technique." This professor who could not make a speech could, however, in conversation make observations of such originality and pungency that they were never forgotten. Sometimes they seemed at first to be irrelevant, but there was always a point worth trying to get. To cite a minor example, when the reviewer was an assistant in Mall's department he once heard Doctor Mall remark, as he came from the dissecting room, "I'd hate to be an archeologist, it's such dirty work"; a saying which seemed a mere jest, but after 21 years is seen to be an indirect way of asserting the dignity and cleanliness of anatomy, which as Miss Sabin points out, he was always very anxious to maintain. Those who knew him longer and in earlier days, no doubt found him less cryptic; and, indeed, it would not be accurate to represent him in this light alone, for his clear, direct and sensible papers on the teaching of anatomy have been like a mariner's chart to many of us; given a hearer whom he appreciated, he could teach in that manner likewise (though not to an audience); and he often so favored even the younger of his disciples.

The accomplishments of such a man, working for his ideals without the least public display, can only be evaluated by those who were close to him and to the movements which he guided. It is clear, as Miss Sabin well shows, that Mall took the lead in reforming anatomical instruction in the United States, doing more than any other teacher to broaden and dignify the subject, and long anticipating the 'project method' of recent educational theory. The chapters entitled 'Inductive teaching' and 'Teacher-investigator,' perhaps the heart of the biography, give many interesting lights on his views of educational liberality, the 'concentration' method, elective teaching, the value of routine examinations, and other of our ever-present pedagogical problems. The chapter which tells of his research students cannot deal completely with the subject at this time, because most of the people who owe their inspiration and training to Mall are still in active service; the list is a long one, and the place these men hold in our scientific community seems to promise him immortality as a teacher of teachers. The reviewer may freely

say one thing, however, which Doctor Sabin does not, namely, that Mall's influence in this respect was partly mediated through others; he managed to find and hold those whose talents complemented his own—people such as Doctor Sabin herself—and these colleagues had their own share in the extraordinary spirit of enthusiasm and enterprise which pervaded his laboratory.

The chapter which deals with the founding of The American Journal of Anatomy and The Anatomical Record cannot fail to interest the readers of this journal. These periodicals were published from his own office for several years, and during that time had a character and style all their own. As a member and officer of The American Association of Anatomists his opinions were always interesting, because he thought much about the problems of organization and control of such societies, and had a viewpoint which curiously mingled democracy with shrewd practicality.

In the final chapters on 'Medicine as science' and 'Research institutes,' Miss Sabin describes Mall's part in certain movements which are still unfinished or under experiment. To some it may seem that he is given too much credit for the plan for full-time clinical teaching now in effect at Johns Hopkins and (in various modifications) in other schools. Ample evidence is given, however, to show that much of the idea was his in the days when it was merely a bold dream; and those who knew the subtle force of his quiet, behind-the-scenes influence can well credit him with a large share in ideas which others were gifted to advance by public exposition.

In the later years of his relatively brief career Mall was much interested in a number of enterprises of cooperative research or partaking of the nature of research institutes. His own institute, the Carnegie Embryological Laboratory, was well established at the time of his death, and its success is obvious; but the other enterprises mentioned in Doctor Sabin's chapter on the subject, in which he was an adviser, either took directions not foreseen by him or did not survive the times of international confusion. If there is something of anti-climax in this part of the story, it is due to the abrupt cessation of Doctor Mall's work at the age of fifty-four.

In short, every stage of Mall's life as recounted in Doctor Sabin's biography contains interest and instruction for everyone engaged in the study of anatomy or medicine, and especially for any who aspire to make a serious contribution to medical education. To read it is to understand and appreciate not only the life of a great and original teacher, but the whole struggle for high standards in American scientific medicine.

George W. Corner.